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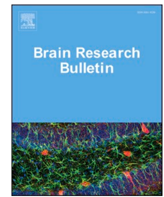

Research report

Gut microbiome composition changes in obstructive sleep apnoea syndrome also in relation to excessive daytime sleepiness

Mariana Fernandes [a,1], Orazio Palmieri [b,1], Stefano Castellana [c,1], Matteo Spanetta [a], Tiziana Latiano [b], Clementina Lupo [a], Claudia De Masi [d], Christian Cardile [a], Carmen Calvello [a], Francesca Izzi [d], Fabio Placidi [a,d], Tommaso Mazza [c], Nicola Biagio Mercuri [a,d], Anna Latiano [b,2], Claudio Liguori [a,d,*,2,3]

[a] *Department of Systems Medicine, University of Rome Tor Vergata, Rome, Italy*
[b] *Division of Gastroenterology, Fondazione IRCCS "Casa Sollievo della Sofferenza", San Giovanni Rotondo, Italy*
[c] *Bioinformatics Unit, Fondazione IRCCS "Casa Sollievo della Sofferenza", San Giovanni Rotondo, Italy*
[d] *Neurology Unit, University Hospital of Rome Tor Vergata, Rome, Italy*

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ABSTRACT

Introduction: Obstructive sleep apnoea syndrome (OSAS) is considered a risk factor for several comorbidities. Alteration in gut microbiome was documented in OSAS animal models and in paediatric patients. This study analysed gut microbiome composition in adult patients with OSAS compared to healthy controls. Further, the effect of excessive daytime sleepiness (EDS) on gut microbiome was evaluated.
Methods: Adult patients with OSAS underwent polysomnographic recording and completed the Epworth Sleepiness Scale (ESS) to assess EDS. Faecal samples were collected and compared between patients and healthy controls. Composition, community diversity, differences in taxa abundance profiles and sample dysbiosis were evaluated through 16S metagenomics and multiple bioinformatics algorithms. OSAS patients were distributed in two groups according to EDS (ESS score≥10) to assess differences in clinical, polysomnographic and faecal data.
Results: Twenty-three OSAS patients were compared to 44 healthy controls. Patients presented significant differences of gut microbiome biodiversity, specifically in qualitative alpha diversity metrics (Faith's PD Kruskal-Wallis test, p-value=0.003; Number_of_Observed_Features, p-value =0.001). OSAS patients tend to cluster together, at least for Jaccard and Unweighted UniFrac distance-based PERMANOVA tests (q-values=0.02 and =0.003, respectively). Several taxa were detected as different in abundance between OSAS patients and healthy controls, although, globally, OSAS patients cannot be considered as "dysbiotic". Differences in bacteria composition were evident between OSAS patients with and those without EDS.
Conclusions: OSAS is associated with gut microbiome alteration in adult patients. EDS in OSAS seems to characterize a different gut microbiome composition, although it can be only hypothesized a gut-mediated effect on EDS in OSAS.

1. Introduction

Obstructive sleep apnoea syndrome (OSAS) is one of the most common sleep disorders, and is characterized by episodes of partial or complete upper airway obstructions, leading to intermittent hypoxia (IH), microarousals, sleep fragmentation, and excessive daytime sleepiness (EDS) (Ryan and Bradley, 2005).

Over the past decade, increasing attention has been given to the role of the gut microbiome in regulating sleep, highlighting a bi-directional relationship between sleep and gut microbiome (Cai et al., 2021; Neroni et al., 2021; Tang et al., 2024). Studies in mouse models of sleep apnea showed that IH leads to significant imbalanced gut microbiota

* Correspondence to: Department of Systems Medicine, University of Rome "Tor Vergata", Via Montpellier 1, Rome 00133, Italy.
E-mail address: dott.claudioliguori@yahoo.it (C. Liguori).
[1] Equally contributed to this work as co-first author
[2] Equally contributed to this work as co-last author
[3] https://orcid.org/0000-0003-2845-1332

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diversity, with a loss of beneficial microbial composition and an expansion of pathogenic microbes, termed dysbiosis. This imbalance may be partially reversible with the restoration of normal oxygen levels (Castro-Grattoni et al., 2016; Moreno-Indias et al., 2016, 2015). Furthermore, IH has been associated with elevated levels of inflammatory mediators, such as IL-6, contributing to systemic inflammation and intestinal hyperpermeability (Heizati et al., 2017). Consistently, although human studies are still limited, findings in paediatric OSAS patients confirmed a reduction in gut microbiota diversity along with an increased presence of gut-barrier-disrupting strains (Collado et al., 2019; Valentini et al., 2020). Consistently, two clinical studies in adult patients with OSAS reported the alteration of gut microbiota profile (Ko et al., 2019; Wang et al., 2022). Specifically, these studies showed that OSAS is associated with a decline in short-chain fatty acid (SCFA)-producing bacteria, such as *Faecalibacterium*, *Bifidobacterium*, *Lactobacillus*, and *Bacteroides*, alongside an increase in pathogenic taxa like *Prevotella*, which has been linked to systemic inflammation (Ko et al., 2019; Wang et al., 2022). These findings suggested that gut microbiome alteration in OSAS patients may contribute to broader, systemic effects, including hypertension and obesity (Cai et al., 2021).

Despite substantial research in animal models (Badran et al., 2020; Khalyfa et al., 2021; Yan et al., 2024), there is a lack of studies investigating gut microbiome changes in adult OSAS patients. Hence, our study aimed to evaluate the gut microbiome composition in adult OSAS patients compared to healthy controls (HC). Additionally, this study investigated the effect of EDS on gut microbiome composition, given the ongoing debate about the mechanisms underlying this disabling symptom in OSAS patients and the absence of correlation between OSAS severity and EDS.

2. Methods

2.1. Participants and Study Procedures

The study included 23 adult OSAS patients (69.57 % male, mean age 50.0 ± 10.11 years) and 44 HC (54.55 % male, mean age 47.5 ± 10.9 years). Patients were recruited from the Sleep Medicine Centre of the University Hospital of Rome Tor Vergata between May 2021 and May 2022. All patients underwent standard neurological and sleep medicine visits, including the Epworth Sleepiness Scale (ESS), polysomnography (PSG), and faecal sample collection. Moreover, faecal samples from a group of HC, which were recruited from the same hospital and the IRCCS "Casa Sollievo della Sofferenza" Hospital, comprising non-hospitalized individuals, laboratory staff, and community donors of stool for faecal microbiota transplantation. Their healthy status was verified using a checklist of health problems and a sleep anamnestic interview to assess sleep habits and sleep apnoea symptoms. None of the HCs had a history of gastrointestinal disease or malignancy.

Inclusion criteria for both groups included no neurological or psychiatric diseases, no concomitant sleep disorders, no CNS-active drugs, and no use of antibiotic, probiotic, supplements or anti-inflammatory for at least 3 months before sampling. Exclusion criteria encompassed systemic, infectious, inflammatory, or autoimmune diseases, heavy smoking, respiratory conditions, cognitive impairment, substance abuse, inflammatory bowel disease, allergies, and any known acute or chronic inflammatory disease. Patients with hypertension or diabetes were also excluded unless their systolic blood pressure was < 140 mmHg, diastolic blood pressure < 85 mmHg, and fasting blood glucose < 100 mg/dL. Additionally, OSAS patients had not started treatment for OSAS at the time of the assessments.

The Ethical Committee of the University Hospital of Rome Tor Vergata approved the study and written informed consent was obtained from all participants.

2.2. Polysomnography

All OSAS patients underwent ambulatory PSG (SOMNOscreen, SOMNOmedics GmbH, Randersacker, Germany) to assess nocturnal sleep while maintaining usual sleep habits, following the procedure described in a previous study (Romigi et al., 2014). Sleep analysis was performed according to the standard criteria and standard parameters were computed (Iber et al., 2007).

2.3. Epworth Sleepiness Scale

EDS was assessed in OSAS patients and HC using the Italian version of the ESS (Vignatelli et al., 2003). Participants rated their likelihood of dozing off during eight activities on a 4-point scale (0−3). The ESS score ranges from 0 to 24, with a score ≥ 10 indicating EDS. All HC had ESS scores below 10, so group comparisons were only made within OSAS patients, dividing them into those with EDS (ESS≥10) and those without EDS (ESS<10).

2.3.1. Faecal sample collection

Stool samples were collected from both OSAS patients and HC. OSAS patients provided samples the morning after their PSG recording. Each sample, collected in a sterile stool tube, was transported in refrigerated containers and stored at −80°C within 8 hours. Samples from the University Hospital of Rome Tor Vergata were shipped on dry ice to the IRCCS "Casa Sollievo della Sofferenza" Hospital, remaining frozen solid upon arrival and stored at −80°C until analysis.

2.4. Laboratory procedures

Total bacterial genomic DNA was extracted by using the QIAamp Power Faecal Kit (Qiagen, Hilden, Germany), as previously described (Andriulli et al., 2022).

Briefly, DNA was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc., Somerset, NJ, USA). The microbial composition of faecal samples was determined using the V3 to V4 hypervariable region of the 16S rRNA gene, according to the Illumina 16S Metagenomic Sequencing Library preparation guide (Part # 15044223 Rev.B; https://support.illumina.com/documents/documentation/chemistry_documentation/16 s/16s-metagenomic-library-prep-guide-15044223-b.pdf) by using Taq Phusion High-Fidelity (Thermo Fisher Scientific, Sunnyvale, CA, USA) in 25 μL reaction volumes.

The amplicons were purified using AMPure XP beads (Beckman Coulter, Milan, Italy) and the ligation of the dual indexing adapters was performed in the presence of Nextera XT Index Primer 1 and Primer 2 (Illumina, San Diego, CA, USA), Taq Phusion High-Fidelity (Thermo Fisher Scientific) and 5 μL of purified DNA, according to the manufacturer's instructions. The products were purified using AMPure XP beads to create the cDNA library. Library concentrations and fragment sizes were measured using Qubit dsDNA BR Assay System (Thermo Fisher Scientific) and an Agilent 2200 TapeStation Bioanalyzer (HS D1000 ScreenTape Assays; Agilent Technologies, Santa Clara, CA, USA), respectively.

2.5. Statistical analysis

Descriptive statistics were computed for sex, age, body max index (BMI) and EDS. Data normality was tested using the Shapiro-Wilk test. Mann-Whitney tests evaluated differences in demographic, clinical, and PSG data between OSAS patients with and without EDS. Statistical significance was set at p-value< 0.05, and the analysis was conducted with SPSS version 25 (IBM, 2020).

2.6. Bioinformatics and statistical data analyses

Sequences, or reads, were initially quality-checked by using the

FastQC Application (Andrews, 2010). Then, the core metagenomics analysis was performed with QIIME2 v.2021.11 suite (Estaki et al., 2020). The QIIME2 DADA2 denoise-paired command was used to process paired-end sequencing data. Forward and reverse reads were truncated to 250 bp (–p-trunc-len-f and –p-trunc-len-r), and the first 10 bases were trimmed from both ends of the reads (–p-trim-left-f and –p-trim-left-r) to remove low-quality regions. These steps ensure high-quality sequences for downstream analysis. The subsequent reads were filtered (by read average quality), end-joined and depleted of chimeric sequences using the QIIME2 integrated DADA2 module (Callahan et al., 2016) Filtered sequences were aligned using the Mafft aligner, and rooted and unrooted 16S phylogenetic trees were constructed using the FastTree algorithm (Price et al., 2009). Taxonomic assignment was obtained by using the Naïve Bayes fitted classifier, pre-trained on the most recent Greengenes reference database (ver. 13.8) (McDonald et al., 2012). Microbial diversity was evaluated via rarefaction curves using the "diversity" module of QIIME. To determine an optimal sampling depth the raw feature table and its corresponding phylogenetic tree were used to generate several rarefaction tests at different read depth cutoffs (10000–25000 reads).

Shannon's diversity index (Shannon et al., 1950), Number of Observed Features, Faith's Phylogenetic Diversity (Faith, 1992), and Pielou's evenness (Pielou, 1967) were computed for each sequence pool and compared using the Kruskal-Wallis test. Sample dissimilarity, Jaccard (Jaccard, 1908), Bray–Curtis (Sorensen, 1948), unweighted UniFrac, and weighted UniFrac distances (Lozupone and Knight, 2005), were visually evaluated through Principal Coordinate Analysis (PCoA) plots using the EMPeror web application (Vázquez-Baeza et al., 2013) available at https://view.qiime2.org. Beta diversity differences across sample groups were evaluated by the permutational multivariate analysis of variance (PERMANOVA) test.

To analyse the differential abundance of microbial taxa between two groups, various methods were employed due to the compositional nature of microbiome datasets. Initially, the QIIME2 ANCOM module was applied after removing contaminants (i.e., mitochondrial/chloroplast origin) and ultra-rare features (present in fewer than 5 samples or with fewer than 20 counts across all samples), resulting in a filtered feature table. Next, feature selection was performed on this table collapsed at the genus level using three R packages: Coda-lasso (Lu et al., 2019), Clr-lasso (Zou and Hastie, 2005), and Selbal (Rivera-Pinto et al., 2018), as described in https://malucalle.github.io/Microbiome-Variable-Selection/.

For predicting genetic and functional content, Picrust v2.3.0b software (Douglas et al., 2020) was used, with the filtered feature table and representative sequences as inputs. The focus was on the unstratified pathway abundance table, using ALDEx2 (Fernandes et al., 2014) to detect variations between groups. ALDEX was run considering three significance cut-offs: 0.5, 1, and 1.5 (absolute value) to identify which predicted pathways were significantly over or down- represented in the OSAS group. Microbial dysbiosis in OSAS samples was further explored in comparison to the HC group using two further methods. First, Bray-Curtis dissimilarities (Lloyd-Price et al., 2019) were calculated from QIIME2 diversity output reports, with the 90th percentile value of HC dissimilarity distributions used to define the "dysbiosis" threshold. Second, the "CLOUD" test (Montassier et al., 2018) was implemented to identify OSAS samples that were ecologically distant from HC samples, testing various reference subset sizes (5 %, 15 %, and 30 %). The abovementioned Bray-Curtis dissimilarity matrix from QIIME2 was used as input data. CLOUD test generates an "r" statistics, i.e., a ratio between sample-to-reference subset neighbourhood diameter and the average neighbourhood diameter for HC set. Data parsing and dysbiosis tests were conducted using the R packages Microbiome v1.8.0 (Lahti and Shetty, 2019) and Phyloseq (McMurdie and Holmes, 2013) v1.30.0.

3. Results

Demographic and clinical data of patients and HCs are presented in Table 1, showing that HCs had lower BMI and ESS total scores compared to OSAS patients. The OSAS group had an ESS mean score of 8.91 ± 5.55, with 11 patients (47.82 %) reporting EDS (ESS ≥ 10). No significant differences were observed in demographic, clinical, and PSG data between OSAS patients with and without EDS (see Table 2).

3.1. 16S rRNA V3-V4 region sequencing results

The QIIME2 DADA2 denoise-paired command was used to process paired-end sequencing data. After clustering, chimaeras removal and filtering, the median sequencing output was 58853 reads (IQR 44507–92262; Section 1.2. "Feature distribution per sample ("per sample" column) and global feature distribution ("per feature")" of Suppl. File 1). The number of high-quality sequence counts ranges from 25730 to 221520, while feature abundance ranges from 1 to 199935 counts as shown in Supplementary File 1.

Using the raw feature table and its corresponding phylogenetic tree, several rarefaction tests were conducted at different read depth cutoffs (refer to Suppl. File S1). This allowed determining an optimal sampling depth with minimal sample loss. A rarefaction depth of 20,000 reads ensured a stable distribution of the alpha diversity metrics across all the investigated groups.

3.2. Faecal microbiota diversity analysis

The degree of dysbiosis among the samples is shown in Suppl. File S1, displaying data on all the OSAS patients at baseline as compared to HCs. According to the results of the Lloyd–Price test, 1 of the 23 OSAS patients showed a marked degree of dysbiosis compared with the HC group (dysbiosis cut-off = 0.92).

Alpha diversity distribution differed between groups at Faith's Phylogenetic Distance (q-value = 0.003) (Fig. 1 and Table 3) and Number of Observed Features (q-value = 0.0098) (Table 3), but not at Shannon's Entropy (q-value = 0.58) and Pielou's evenness (q-value = 0.11).

According to the PERMANOVA test, OSAS samples clustered separately from HC samples, with significant differences in Jaccard similarity (Pairwise PERMANOVA q-value=0.02) and Unweighted UniFrac dissimilarity (q-value=0.003), but not in Bray-Curtis (q-value=0.27) or Weighted UniFrac (q-value=0.40) indexes (Table 4). Full results for diversity analysis are reported in Supplementary File 1.

3.3. Microbial signature detection

From the QIIME2 ANCOM modules, run at Genus ("L6") and Species ("L7") levels, no specific organisms associated with OSAS patients were underlined. Differences in microbial composition between the two groups were detected using Coda-lasso, Clr-lasso and Selbal methods. The software was set to search for a maximum of 20 discriminant taxa (between OSAS and HC groups) across the whole feature table (shown in

Table 1
Participants' demographic and clinical data.

	OSAS patients (n = 23)	Healthy Controls (n = 44)	p-value
Mean age (years)	50.0 ± 10.11	47.5 ± 10.9	0.496
Sex, n (%)	16 (69.57 %)	24 (54.55 %)	0.354
Male	7 (32.43 %)	20 (45.45 %)	
Female			
BMI (Kg/m^2)	27.93 ± 4.50	25.51 ± 4.19	0.049
ESS score	8.91 ± 5.55	5.53 ± 3.06	0.018

Abbreviations: BMI, Body mass index; ESS, Epworth Sleepiness Scale; OSAS, Obstructive sleep apnea syndrome; Values are expressed as means ± SD.

Table 2
Demographic, clinical, and polysomnographic data in OSAS patients with EDS and without EDS.

	OSAS patients (n = 23)	OSAS patients with EDS (n = 11)		OSAS patients without EDS (n = 12)		Test Differences	
	Mean ± SD	Median	25–75th Percentile	Median	25–75th Percentile	U / χ2	p-value
Demographic and Clinical data							
Mean age (years)	50.0 ± 10.11	46.00	43.00 – 54.00	52.50	48.25 – 57.00	42.50	0.151
Sex, n (%)	16 (69.57 %)	8 (72.7 %)		8 (66.7 %)		0.100	0.752
Male	7 (32.43 %)	3 (27.3 %)		4 (33.3 %)			
Female							
BMI (Kg/m^2)	27.93 ± 4.50	27.30	23.1 – 31.80	29.37	22.98– 31.37	67.50	0.928
Polysomnographic data							
Total Time in Bed (min)	453.58 ± 62.07	443.02	402.00 – 516.30	446.61	413.34 – 517.95	64.00	0.928
Total sleep time (min)	383.04 ± 68.04	416.00	264.50 – 447.00	384.50	367.38 – 418.88	76.0	0.566
Sleep efficiency (%)	84.32 ± 9.19	87.30	76.80 – 94.50	85.30	77.60 – 91.05	71.00	0.786
Sleep latency (min)	5.44 ± 3.96	3.80	2.70 – 6.20	5.20	2.18 – 11.15	54.50	0.487
REM sleep latency (min)	86.87 ± 47.00	90.50	57.50 – 105.00	78.00	51.88 – 107.13	78.00	0.487
WASO (min)	68.84 ± 38.66	67.80	24.52 – 119.80	69.47	39.56 – 94.21	61.50	0.786
REM (%)	17.96 ± 5.54	18.70	15.10 – 22.50	17.00	15.10 – 18.83	76.50	0.525
N1 (%)	4.50 ± 2.86	4.00	2.50 – 5.50	3.80	2.60 – 6.18	64.50	0.928
N2 (%)	56.88 ± 7.39	54.40	51.70 – 56.10	56.40	51.60 – 66.60	49.50	0.316
N3 (%)	20.68 ± 7.43	23.00	18.40 – 25.00	20.95	14.60 – 26.73	71.50	0.740
AHI	31.85 ± 19.52	42.40	25.50 – 50.60	23.60	17.20 – 47.80	60.50	0.118
Mean SpO2 *(%)*	93.78 ± 1.76	94.00	91.00 – 95.00	94.50	93.25 – 95.00	58.00	0.651
ODI	25.68 ± 18.19	26.20	14.70 – 41.90	22.00	12.80 – 39.95	56.00	0.288

Abbreviations: BMI, Body mass index; EDS, Excessive daytime sleepiness; OSAS, Obstructive sleep apnea syndrome; N1, stage 1 of non-REM sleep; N2, stage 2 of non-REM sleep; N3, stage 3 of non-REM sleep; REM, rapid eye movements; WASO, wake after sleep onset; AHI, apnoea-hypopnea index; SpO2, oxygen saturation; ODI, oxygen desaturation index.

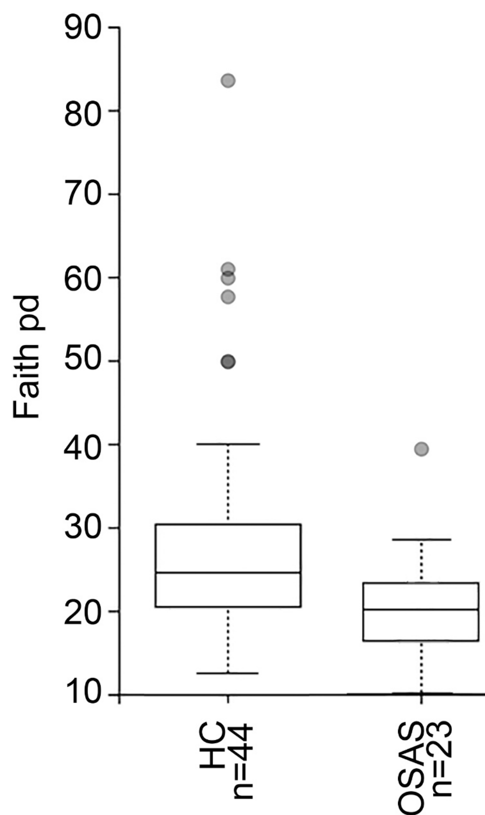

Fig. 1. Group-Specific Faith's Phylogenetic Distance. Boxplot showing Faith's Phylogenetic Distance, a measure of alpha diversity, across study groups. The median, interquartile range (IQR), and outliers are displayed. Kruskal–Wallis test (all groups): q value = 0.0034. See Table 4 and Supplementary_File_1 for details.

Suppl. File 2, Feat Selection, sheet 2). Global results from each tool are summarized in Suppl. File 2. Five taxa resulted to be the most abundant in patients: *g_Clostridium from Erysipelotrichaceae family, g_Megasphaera, g_Catenibacterium, g_Butyricicoccus, and an unknown genus from the OD1 phylum*; four taxa were instead the most abundant in HC: *g_Clostridium from Lachnospiraceae family, g_SMB53, an unknown genus from f_Desulfovibrionaceae, and an unknown genus from RF32 order*. Three taxa were consistently identified by the three implemented methods, i.e., Coda-lasso, Clr-lasso and Selbal, in HCs and OSAS patients with and without EDS: the *Anaerotruncus* genus was more abundant in OSAS patients with EDS, while *Clostridium* genus (from the *Erysipelotrichaceae* family) and an unclassified group were higher in OSAS patients without EDS. Additionally, four taxa were more abundant in the HC group. The comparison between HCs and OSAS patients resulted with a few distinct genera: *Clostridium* (from the *Lachnospiraceae* family), and an unknown *Clostridia genus* in OSAS with EDS, and an unknown *Desulfovibrionaceae genus* and *SMB53* in OSAS without EDS.

Table 3
Summary of pairwise group comparisons for four alpha diversity indexes.

	OSAS vs. Healthy Controls
Shannon's Entropy	0.58
Pielou's evenness	0.11
Number of Observed Features	0.0098
Faith's Phylogenetic Distance	0.0034

Benjamini and Hochberg corrected p-values (q-values) for Kruskal–Wallis tests are shown.

Table 4
Summary of pairwise group comparisons for beta diversity measures.

	OSAS vs. Healthy Controls
Jaccard similarity	0.016
Bray-Curtis dissimilarity	0.27
Unweighted UniFrac dissimilarity	0.003
Weighted UniFrac dissimilarity	0.40

Benjamini and Hochberg corrected p-values (q-values) for PERMANOVA tests are shown.

Pairwise analysis of OSAS patients based on EDS (ESS $\geq$ 10 vs. ESS $<$ 9) revealed three taxa with different abundance levels: *Anaerotruncus* was higher in patients with ESS $\geq$ 10 (thus presenting EDS), while *Eggerthella* and a *Lactobacillales genus* were more abundant in those with ESS $<$ 9 (thus without EDS). The composition of the microbial

communities among the samples can be observed in Suppl. File 1 (Microbial relative abundance), which shows the most represented phyla and the top 20 most represented (relative abundance) genera.

3.4. Differential analysis of predicted functional content

PICRUSt inferred 1664 gene functions (defined by Enzyme Commission identifiers) and 337 metabolic pathways (defined by MetaCyc identifiers). ALDEx2 analysis found no differentially abundant pathways for OSAS, even at the relaxed cut-off (effect > 0.5 or < -0.5; Suppl. File 3).

3.5. Dysbiosis analysis of OSAS patients vs. healthy controls

One OSAS sample was likely dysbiotic (median Bray-Curtis dissimilarity of 0.93, threshold 0.91), with a CLOUD (Montassier et al., 2018), test "r" statistic of 1.39 (i.e., its neighbourhood diameter is 1.39 larger than the average neighbourhood diameter calculated in the control set; $p < 0.05$) ("DS029RM-BASALE", see Suppl. File 3 for details and Suppl. File 1). Another sample ("DS019-BASALE") was weakly dysbiotic (median Bray-Curtis 0.91, CLOUD "r" statistic 0.9, $p = 0.73$).

4. Discussion

In this study, gut microbiome composition and diversity was analysed in adult patients with OSAS compared to a HC group. The alpha diversity analysis revealed significant differences in Faith's phylogenetic distance, indicating lower diversity in the OSAS patients compared to HCs. Similarly, beta diversity, measuring between-sample differences, showed lower values in OSAS patients, with significant results in Jaccard-based and unweighted UniFrac-based diversity, highlighting compositional and phylogenetic abundance differences compared to HCs. Although the significance of the present findings, this study presents preliminary findings require future large investigations implementing this study design with the inclusion of the dietary and lifestyle habits and evaluating the effects of continuous positive airway pressure (CPAP) treatment.

The microbiome alpha diversity analysis documented a significant difference in Faith's phylogenetic distance, which uses phylogenetic trees generated with molecular or cladistic data to estimate diversity. In particular, the gut microbiome composition differs between OSAS patients and HCs since alpha diversity and the number of observed features were significantly lower in the OSAS group. Moreover, beta diversity, which is a measure of between-sample differences of pairs of communities, was also different between OSAS patients and HCs, with lower values in the patient group. Specifically, statistically significant results were evident in the Jaccard-based diversity, which identifies compositional differences, and in the unweighted UniFrac-based diversity, which identifies phylogenetic abundance differences. Considering all these results, the gut microbiome composition of OSAS patients is different from that of HCs at the phylum level. Previous studies conducted in animal models showed that IH, one of the main features of OSAS, produces gut dysbiosis and a state of inflammation damaging the gut wall barrier (Moreno-Indias et al., 2016, 2015). Consistently, two studies in paediatric OSAS patients documented lower microbial diversity and higher abundance of pro-inflammatory bacteria in these patients when compared to healthy children (Collado et al., 2019; Valentini et al., 2020). Therefore, it was hypothesised the condition of "leaky gut" that may also support the etiopathology of systemic consequences of OSAS, such as neuroinflammation, arterial hypertension and metabolic diseases.

In our analysis, significant shifts in gut microbiome composition were observed in OSAS patients compared to HCs, with notable increases in *Erysipelotrichaceae*, *Veillonellaceae*, and *Clostridiaceae*, while *Lachnospiraceae* and *Desulfovibrionaceae* were less abundant. The enrichment of *Erysipelotrichaceae* and *Veillonellaceae* has been previously linked to systemic inflammation, which can be triggered by OSAS, suggesting that these microbial alterations might contribute to the inflammatory burden observed in this sleep disorder. Furthermore, the observed increase in *Clostridiaceae*, a bacterial family associated with pro-inflammatory responses in the gut, aligns with previous findings in paediatric OSAS patients and animal models of IH (Collado et al., 2019; Moreno-Indias et al., 2015). These results reinforce the hypothesis that imbalance in gut microbiota composition could be linked to systemic consequences of OSAS, including neuroinflammation and metabolic dysregulation.

One key finding of our study is the lower number of species and reduced alpha and beta diversity in the OSAS patients compared to HCs, aligning with previous animal model studies (Castro-Grattoni et al., 2016; Moreno-Indias et al., 2016, 2015). Conversely, the studies performed in paediatric OSAS patients only showed a reduction in alpha diversity (Valentini et al., 2020). Noteworthy, the differences between our study and the literature may be due to our focus on adult OSAS patients without comorbidities, which can affect gut microbiome composition. Another significant finding is the difference in the phyla between patients and healthy subjects. Specifically, the *Firmicutes phylum*, including *Erysipelotrichaceae, Veillonellaceae*, and *Ruminococcaceae* families, was more abundant in OSAS samples, while *Lachnospiraceae*, *Clostridiaceae*, and *Desulfovibrionaceae* were less abundant, similar to previous studies (Ko et al., 2019; Wang et al., 2022). This dysregulation in *Firmicutes* may impact sleep quality, as these bacteria produce gamma-aminobutyric acid, an inhibitory neurotransmitter that promotes sleep, and it can be only preliminary hypothesized a mediated effect of gut microbiome diversity on sleep quality of OSAS patients. Moreover, a higher number of *Erysipelotrichaceae* has been documented in OSAS patients. In previous studies this phylum was correlated with both the oxygen saturation parameters during sleep and the sleep fragmentation (Collado et al., 2019), suggesting a link to nocturnal hypoxia. OSAS samples also showed an increase in *Clostridiaceae*, confirming the previous evidence reported in children with OSAS. This bacteria family known to induce inflammation in mouse model (Tripathi et al., 2018), thus reflecting the pro-inflammatory state of OSAS patients.

Our analysis also explored OSAS patients with and without EDS, measured through ESS. Consistently, the mechanisms at the basis of EDS in patients with OSAS are still unclear, since no association was found between EDS and any marker of OSAS severity. Demographic, clinical, and PSG data were indeed similar between the two subgroups of OSAS patients, distributed on the basis of the presence of EDS. However, differences in taxa were detected between HCs, OSAS patients with EDS, and OSAS patients without EDS. *Ruminococcaceae* were more abundant in patients with EDS who showed also a reduction of *Lactobacillales* and *Coriobacteriaceae*, while *Erysipelotrichaceae* were more prevalent in patients without EDS. The modification of these phyla has been previously documented after chronic sleep fragmentation (Poroyko et al., 2016). In particular, chronic sleep deprivation produces gut microbiome changes with the preferential growth of highly fermentative members of *Ruminococcaceae* and a decrease in *Lactobacillaceae* families. This effect leads to systemic and visceral white adipose tissue inflammation and alteration in insulin sensitivity in mice (Poroyko et al., 2016). Finally, *Lachnospiraceae* abundance was higher in patients with EDS compared to HCs. The abundance of *Lachnospiraceae* has been observed in animal studies evaluating the effects of the induced IH condition on gut microbiome (Moreno-Indias et al., 2015).

Our functional prediction using PICRUSt did not show any significant difference at the pathway level. This is probably due to the limited sample size of the patients analysed that actually makes this a full-fledged exploratory study. For this reason, we intend to analyse a larger cohort of OSAS patients in future follow-up studies. Furthermore, it is worth noting that 16S-based metagenomics does not give an in-depth, full picture of the whole genomic content, also considering the microbial gene expression level. Thus, full shotgun metagenomics or meta-transcriptomics are definitely better strategies for microbial

functional profiling.

While our findings indicate a distinct gut microbiome composition in OSAS patients, particularly those with EDS, this study does not provide direct mechanistic evidence for a gut-brain axis involvement. The observed differences in bacterial taxa, including the enrichment of *Erysipelotrichaceae* and *Veillonellaceae* in OSAS patients, align with previous research suggesting a role for gut microbiota in inflammation and metabolic regulation (Cai et al., 2021). However, these results remain associative, and it is unclear whether microbiome alterations are a consequence of OSAS-related phenomena (such as IH and sleep fragmentation) or whether they contribute to disease pathophysiology. To address this gap, longitudinal studies are essential to systematically track microbiome composition over time in OSAS patients, identifying whether microbial shifts precede or follow disease onset and progression. Such studies could help determine whether gut microbiome alteration serves as early indicator or driver for the systemic involvement in OSAS. Additionally, interventional studies aimed at modifying gut microbiota could provide valuable insights into potential therapeutic strategies. Approaches such as probiotic supplementation, targeted dietary modifications, or faecal microbiota transplantation should be explored to assess their impact on OSAS severity and symptoms, particularly EDS. Moreover, the impact of CPAP therapy on gut microbiota composition remains largely unexplored. CPAP is the gold standard treatment for OSAS, yet its potential role in reversing microbiome alterations and reducing inflammation warrants further investigation. Controlled clinical trials could clarify whether microbiome modulation mitigates systemic inflammation, metabolic dysfunction, or neuroinflammatory pathways involved in the sleep-wake regulation. Furthermore, animal models could be leveraged to establish mechanistic links by experimentally altering microbiota composition and evaluating resulting changes in sleep physiology, inflammatory markers, and cognitive outcomes. Expanding research efforts in both clinical and experimental settings is necessary to determine whether gut microbiome modulation represents a viable strategy for OSAS management.

Considering the lack of biomarkers for EDS in patients with OSAS, this finding introduces another important issue that need to be further addressed in future studies. However, the impact of gut microbiome changes on OSAS patients' health remains under investigation. OSAS is associated with increased risks of cardiovascular disorders, diabetes, stroke, neurodegenerative diseases, and obesity (Abbasi et al., 2010). Inflammatory drivers and the heart- or brain-gut axes are proposed mechanisms for these increased risks (Cai et al., 2021). Changes in gut microbiome composition might contribute to the development of these conditions. Therefore, further studies should explore the relationship between OSAS and gut microbiome dysregulation, since its modulation could reduce the risk of development of systemic or neurological disorders.

This study presents some limitations that should be acknowledged. The relatively small sample size, particularly in subgroup analyses (OSAS with and without EDS), may have limited the statistical power to detect subtle differences in gut microbial composition. However, the exploratory nature of this study aimed to address gaps in the literature, as most previous research has focused on paediatric OSAS populations, with limited data on adults. Additionally, this study represents an initial attempt to investigate potential associations between EDS and gut microbiome composition. As a preliminary step, these findings provide a foundation for future research to further explore gut microbial patterns in OSAS. Additionally, while we controlled for major confounders by excluding participants with comorbidities, we did not account for dietary habits and lifestyle factors, which can significantly influence gut microbiota composition. Differences in macronutrient intake may have contributed to the observed microbial shifts. High-fat, low-fiber diets promote *Erysipelotrichaceae* and *Veillonellaceae* (Bailén et al., 2020), taxa enriched in OSAS patients, while fiber-rich diets support *Lachnospiraceae* (Carelli et al., 2023), which showed lower abundance in OSAS group. Additionally, physical inactivity, frequently observed in OSAS, is associated with reduced gut microbial diversity and lower *Lachnospiraceae* levels (Clarke et al., 2014; Clauss et al., 2021). The absence of dietary and lifestyle assessments makes it unclear whether these alterations result from OSAS pathophysiology or pre-existing dietary patterns. Future studies should incorporate detailed dietary assessments and controlled feeding protocols to minimize these potential biases. Another limitation is the use of PICRUSt for functional prediction, which infers microbial functions from reference genomes rather than directly sequencing functional genes. This approach may overlook strain-specific variations and underrepresent key metabolic pathways. To achieve a more detailed understanding of microbial functionality in OSAS, future studies should integrate full-shotgun metagenomics, enabling direct sequencing of microbial genomes for higher-resolution functional profiling. Additionally, meta-transcriptomics could provide insights into active microbial functions by analysing RNA transcripts, while metabolomics could reveal host-microbiome metabolic interactions. These advanced approaches will improve mechanistic understanding and support the development of microbiome-targeted therapeutic strategies. Lastly, while our findings support a potential association between gut microbiome alterations and EDS in OSAS patients, no causal relationship can be inferred from this cross-sectional study. Longitudinal studies with mechanistic approaches are needed to determine whether microbiome changes contribute to disease pathology or are a consequence of OSAS-related physiopathological alterations.

Nonetheless, this is the first study investigating the gut microbiome in a group of adult OSAS patients performing PSG and distributed based on EDS, opening new avenues for research. A major strength is that all OSAS patients included had no comorbidities, such as systemic or neurological disorders, reducing confounding factors that can influence gut microbiome (i.e., diabetes, hypertension, heavy smoking). Furthermore, this selection might explain differences in sleep structure and stages compared to previous literature (Li et al., 2019; Shahveisi et al., 2018), as our study focused on gut microbiome dysregulation appearing earlier than comorbidities frequently seen in OSAS patients. Hence, our study design permitted the enrolment of an OSAS population relatively different from that of other studies, explaining the low degree of sleep macrostructure impairment observed. However, sleep microstructure analysis was not performed, warranting further analysis to address this inconsistency. Another limitation is that OSA diagnosis was based solely on the apnoea-hypopnea index (AHI), without reporting respiratory effort-related arousals or the respiratory disturbance index. Additionally, sleep apnoea screening in the HC group was conducted through a sleep amnestic interview, and the lack of PSG recording in controls limits further evaluations.

5. Conclusion

Our preliminary findings suggest different gut microbiome composition in OSAS patients compared to HCs. Significant differences indicate pathways connecting gut microbiome changes to OSAS features. The unique gut microbiome in OSAS patients with EDS highlights the importance of investigating its role for potentially understanding the causes of EDS in OSAS. Further research is needed to explore therapeutic interventions targeting the gut microbiome to reduce systemic and neurological risks in OSAS patients.

Abbreviations

AHI, Apnoea and hypopnea index
BMI, Body Mass Index
EDS, Excessive Daytime Sleepiness
ESS, Epworth Sleepiness Scale
IH, Intermittent Hypoxia
HC, Healthy controls
OSAS, Obstructive Sleep Apnoea Syndrome
PSG, Polysomnography

N1, Stage 1 of non-REM sleep
N2, Stage 2 of non-REM sleep
N3, Stage 3 of non-REM sleep SL Sleep onset latency
REM, Rapid eye movement
REML, REM sleep latency
SE, Sleep efficiency
TST, Total sleep time
WASO, wakefulness after sleep onset

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CRediT authorship contribution statement

Latiano Anna: Writing – review & editing, Supervision, Investigation, Conceptualization. **Castellana Stefano:** Writing – original draft, Formal analysis. **Liguori Claudio:** Writing – review & editing, Writing – original draft, Supervision, Formal analysis. **Spanetta Matteo:** Investigation. **Latiano Tiziana:** Writing – review & editing. **Lupo Clementina:** Investigation. **De Masi Claudia:** Investigation. **Cardile Christian:** Investigation. **Calvello Carmen:** Investigation. **Izzi Francesca:** Writing – review & editing, Formal analysis. **Placidi Fabio:** Writing – review & editing, Formal analysis. **Mazza Tommaso:** Formal analysis. **Fernandes Mariana:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Mercuri Nicola Biagio:** Writing – review & editing. **Palmieri Orazio:** Writing – original draft.

Declaration of Competing Interest

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.brainresbull.2025.111251.

Data availability

Data will be made available on request.

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