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Gut Microbiome in Patients with Obstructive Sleep Apnoea

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Abstract: Background: Obstructive sleep apnoea (OSA) is a risk factor for cardiovascular disease. Alterations in the gut microbiome have been implicated in the development of cardiovascular disease and may potentially link OSA to its cardiovascular consequences. However, only one study to date has investigated gut microbiomes in adult patients with OSA. Methods: 19 patients with OSA and 20 non-OSA controls participated in the study. Following a diagnostic sleep study, blood was collected for metabolic profiling, and the subjects provided a stool sample for microbiome analysis. The gut microbiome was investigated using the 16S ribosomal RNA method. Results: Patients with OSA had a higher relative abundance of the *Proteobacteria* phylum ($p = 0.03$), *Gammaproteobacteria* class ($p = 0.01$), *Lactobacillae* family ($p = 0.02$), *Lactobacillus* ($p = 0.03$), and *Roseburia* genus ($p = 0.03$), and a lower abundance of the *Actinobacteria* phylum ($p = 0.03$). The abundance of *Proteobacteria*, *Gammaproteobacteria*, *Lactobacillae*, and *Lactobacillus* were related to disease severity and dyslipidaemia (all $p < 0.05$), whilst the abundance of *Proteobacteria* and *Gammaproteobacteria* was also related to hypertension and cardiovascular disease (all $p < 0.05$). However, following adjustment for relevant confounders only the association between OSA and *Actinobacteria* remained significant ($p = 0.04$). Conclusions: Obstructive sleep apnoea is associated with only subtle changes in gut microbiome. Further studies should investigate gut dysbiosis in OSA.

Keywords: gut microbiome; dysbiosis; cardiovascular; comorbidity; sleep apnoea; sleep disorder

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1. Introduction

Obstructive sleep apnoea (OSA) is a chronic disease which is characterised by repetitive collapse of the upper respiratory tract during sleep, resulting in chronic intermittent hypoxaemia and frequent microarousals. OSA is a risk factor for cardiovascular and cerebrovascular disease, with hypertension, dyslipidaemia, and enhanced systemic inflammation and oxidative stress being the most investigated mechanisms linking OSA with its comorbidities [1].

More recently, alterations in the gut microbiome have come into attention as a possible mechanism leading to cardiovascular disease [2]. Certain bacteria may disrupt the gut barrier, facilitating the leakage of bile acids and bacterial toxins, leading to systemic inflammation. In addition, bacterial metabolites such as trimethylamine-N-oxide (TMAO)

can enter circulation. Systemic inflammation and TMAO together can lead to foam-cell formation and atherosclerosis [2]. In addition, gut bacteria may produce precursors for neurotransmitters which could affect sleep quality [3].

Obstructive sleep apnoea may lead to gut dysbiosis via various mechanisms. First, some of the bacteria are oxygen-sensitive [4], and intermittent hypoxaemia in animal models was related to alterations in the gut microbiome and a consequential increase in circulating bacterial toxins and bile acids [3,5,6]. Second, sleep fragmentation in animals and humans is itself also related to gut dysbiosis [3,5,6]. In addition, OSA is related to high fat and high carbohydrate intake [7] which can alter the composition of the gut microbiome [8] and is associated with a pro-atherosclerotic microbiome profile [2].

Interestingly, the number of studies investigating gut microbiomes in patients with OSA is surprisingly small [9–11], with only one focusing on adults [11]. Analysing young (2-year-old) snorer and non-snorer children, Collado et al. reported an increased Firmicutes/Bacteroides ratio and a reduced Actinobacteria/Proteobacteria ratio which were hypothesised to relate to later development of comorbidities [9]. In a small (n = 15) pilot study conducted in children (2–12 years old), Valentini et al. reported decreased microbial diversity and an increase in gut-barrier-disrupting microbial strains, such as *Proteobacteria* [10]. In adults, Ko et al. reported no difference in relative abundance at the phylum level, but they found significant differences in various genera between the OSA and control groups [11]. Most particularly, they reported a decreased abundance of short-chain fatty-acid-producing bacteria, which could relate to disruption of the gut barrier and consequential increased systemic inflammation [11]. Another report from the same study described that the *Prevotella* enterotype was the most strongly related to OSA [12]. Of note, the only study in adult humans was conducted in Asian people [11]. As diet is a main factor impacting on the composition of gut microbiome [8,13], these findings cannot be necessarily translated to a Western population.

The aim of this study was to analyse gut microbiomes in patients with OSA and non-OSA controls, and to compare the results with markers of disease severity and comorbidities.

2. Materials and Methods

2.1. Study Design and Subjects

Caucasian subjects participating in the sleep cohort [14] of the Hungarian Twin Registry [15] who agreed to provide a stool sample for microbiome analysis were included. None of the participants were diagnosed with OSA prior to the study or had ever had any treatment for OSA. Only one member of the twin pair was selected following randomisation using an online platform (https://www.sealedenvelope.com/simple-randomiser/v1/lists (accessed on 26 September 2021). Subjects with acute respiratory, heart, and renal failure, as well as inflammatory bowel disease, were excluded. As part of the study protocol [14], blood pressure was measured in the morning and venous blood was collected for serum glucose, total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglycerides, and C-reactive protein (CRP) measurements. The comorbidities were defined according to medical history, available hospital reports, and drug charts, as well as blood pressure measurements and blood results.

This study was conducted in accordance with the Declaration of Helsinki and approved by the local Ethics Committee (Semmelweis University TUKEB 30/2014 and TUKEB 189/2014) and all participants gave their informed consent.

2.2. Sleep Studies

Cardiorespiratory polygraphy (n = 13) and polysomnography (n = 26) were conducted with the Somnoscreen Plus Tele PSG and the Somnoscreen RC devices (Somnomedics GmbH, Randersacker, Germany). Sleep stages, movements, and cardiopulmonary events were scored manually according to the American Academy of Sleep Medicine guidelines [16]. Apnoea was defined as $\geq$90% drop in the nasal flow lasting for $\geq$10 s. Hypopnoea was defined as $\geq$30% drop in the nasal flow lasting for $\geq$10 s, which is associated

with either ≥3% drop in oxygen saturation (on both PG and PSG) or arousal (on PSG). Apnoea-hypopnoea index (AHI), oxygen desaturation index (ODI), and the percentage of total sleep time spent with saturation below 90% (TST90%) were calculated. OSA was diagnosed if AHI was ≥5/h.

2.3. Stool Sample Collection and Analysis

The stool sample collection and analysis followed the protocol published previously [17]. Briefly, following DNA extraction, we performed library extraction for the hypervariable region V3–V4 of microbial 16S ribosomal RNA. Libraries labelled with individual index pairs and validated with Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) were sequenced after pooling on an Illumina MiSeq platform by running a 600-cycle MiSeq Reagent Kit v3 (Thermo Fischer Scientific, Waltham, MA, USA).

The quality of raw reads was assessed with FastQC and MultiQC [18]; the low-quality sequences were filtered and trimmed with Trimmonmatic [19], and we kept only sequences with a minimal length of 50. The low-quality and the first 12 base calls were discarded (Phred score < 20, sliding window size 5). The read classification was performed with a Kraken2 [20], with k-mer size 31 against the SSU Ref NR 99 database (release 132) of SILVA [21]. The microbiome composition and taxa abundances were estimated with Bracken [22].

The microbiome data were further processed as follows. The taxa with low abundance (present maximum of 10% in the OSA or control groups) and with low support (if at maximum 100 reads were classified as that taxa) were discarded. After the filtering process, a Bayesian-multiplicative replacement of zeros was carried out using the zCompositions R package [23], which was followed by a centred log-ratio (CLR) transformation of count and ratio values as implemented in scikit-bio 0.5.6 [24]. The Simpson's, Shannon, and richness alpha diversity metrics were calculated on the filtered and pre-processed taxonomic composition table. The composition similarities were investigated with PERMANOVA [25]. To obtain potential biomarker candidates for OSA, we performed LASSO analysis as implemented in scikit-learn 1.0.2. The optimal hyperparameters were selected via 5-fold cross-validation procedure using ROC-AUC as target metric.

The distribution of the clinical data and relative abundances were analysed with the Shapiro–Wilk test. The OSA and control groups were compared with Mann–Whitney and t-tests. The relative abundances of bacteria were correlated to clinical variables with Mann–Whitney and Spearman tests. The comparison in the microbiome data between the OSA and control groups was adjusted for age, gender, BMI, HDL-C, and triglyceride using multivariate logistic regression analysis. The JASP 0.14 (JASP Team, University of Amsterdam, Amsterdam, The Netherlands) software was used for statistical analysis.

Data are presented as mean ± standard deviation or median/interquartile range/. A p-value < 0.05 was considered significant.

As the results were obtained from a limited population, no formal power calculations were performed. Post hoc sensitivity analyses demonstrated that we could detect larger differences between the two groups than an effect size of 0.95 with a power of 0.80 and an α error probability of 0.05 [26].

3. Results

3.1. Comparison of the OSA and Control Groups

Nineteen subjects were diagnosed with OSA. Patients with OSA were older, had a higher prevalence of dyslipidaemia, had lower HDL-C levels, and higher AHI, ODI, and TST 90% values. The comparison of the groups is summarised in Table 1.

AHI—apnoea-hypopnoea index, BMI—body mass index, CRP—C-reactive protein, DBP—diastolic blood pressure, ESS—Epworth Sleepiness Scale, HDL-C—high-density lipoprotein-cholesterol, LDL-C—low-density lipoprotein-cholesterol, ODI—oxygen desaturation index, SBP—systolic blood pressure, SPT—sleep period time, TST—total sleep time, TST90%—percentage of total sleep time spent with saturation below 90%

Table 1. Descriptive analysis of the OSA and control groups.

	OSA (n = 19)	Control (n = 20)	p
Age (years)	55 ± 12	43 ± 16	0.01
Gender (males%)	53	35	0.27
BMI (kg/m^2)	26.3/25.0–28.1/	22.8/20.9–27.1/	0.07
Smoking (ever%)	32	10	0.09
Hypertension (%)	53	35	0.27
Cardiovascular disease (%)	11	5	0.52
Arrhythmia (%)	21	10	0.34
Diabetes (%)	11	0	0.14
Dyslipidaemia (%)	57	25	0.04
SBP (mmHg)	135/118–140/	120/120–130/	0.39
DBP (mmHg)	80/70–90/	75/70–80/	0.06
Fasting blood glucose (mmol/L)	4.8/4.4–5.3/	4.4/4.1–5.1/	0.32
CRP (mg/L)	1.8/1.2–4.1/	1.6/0.7–4.7/	0.66
Total cholesterol (mmol/L)	5.5 ± 1.1	5.4 ± 1.1	0.76
LDL-C (mmol/L)	1.0 ± 1.7	1.0 ± 1.1	0.86
HDL-C (mmol/L)	1.2/0.9–1.8/	1.7/1.3–2.1/	0.04
Triglyceride (mmol/L)	1.7 ± 0.6	1.1 ± 0.4	<0.01
TST (min)	388/361–423/	383/357–412/	0.56
SPT (min)	429/413–450/	419/397–435/	0.59
Sleep%	93/89–97/	95/85–99/	0.66
AHI (1/h)	8.8/6.5–12.2/	1.8/0.9–2.6/	<0.01
ODI (1/h)	6.9/5.2–9.9/	0.7/0.2–1.2/	<0.01
TST90%	0.7/0.2–2.2/	0.0/0.0–0.0/	<0.01
ESS	6.9 ± 3.5	6.8 ± 3.5	0.93

Around half of the participants did not take any medications. Patients in the OSA group tended to take more ACE inhibitors than controls (p = 0.04), but there was no difference in any of the other medications (Table 2).

3.2. Comparison of the OSA and Control Groups Using Bioinformatics Analysis

Using the centred log ratio data, we performed the LASSO procedure, which resulted in selecting a small subset of microbes in each taxon (see Supplementary Material for the exact species selected). Using this subset, we constructed correlation heatmaps to investigate structural differences between patients with OSA controls at different taxonomic levels. Spearman correlations were used; clustering on the heatmaps was achieved using the Euclidian distance measure. In general, we did not notice major differences in the correlation structure of the data between the examined groups; however, there were some minor findings, especially at the genus level (Figures 1 and 2). In both figures, a clear positively correlated cluster can be identified, mainly composed of genera of the *Prevotellaceae* family as well as the *Porphyromonas* and *Lachnosporaceae* genera. However, the correlations, especially with the *Porphyromonas* genus, are much clearer in the controls, which may signal a disbalance of flora in the OSA group.

Table 2. List of medications in each group.

	OSA (n = 19)	Control (n = 20)	p
Not on any medications (%)	47	45	0.86
ACE inhibitor (%)	37	10	0.04
Angiotensin Receptor Blocker (%)	0	5	0.32
Beta-blocker (%)	16	10	0.59
Calcium chanel blocker (%)	10	0	0.13
Diuretic (%)	10	5	0.52
Clopidogrel (%)	10	5	0.52
Aspirin (%)	5	0	0.30
Statin (%)	21	5	0.13
Antidepressant (%)	5	5	0.97
Benzodiazepines (%)	10	5	0.52
Allopurinol (%)	5	0	0.30
Antidiabetic (%)	5	0	0.30
L-thyroxine (%)	5	15	0.32
Hormonal replacement therapy (%)	0	5	0.32
Laxatives (%)	5	5	0.97
Antihistamine (%)	0	15	0.08
Proton pump inhibitor (%)	15	5	0.27

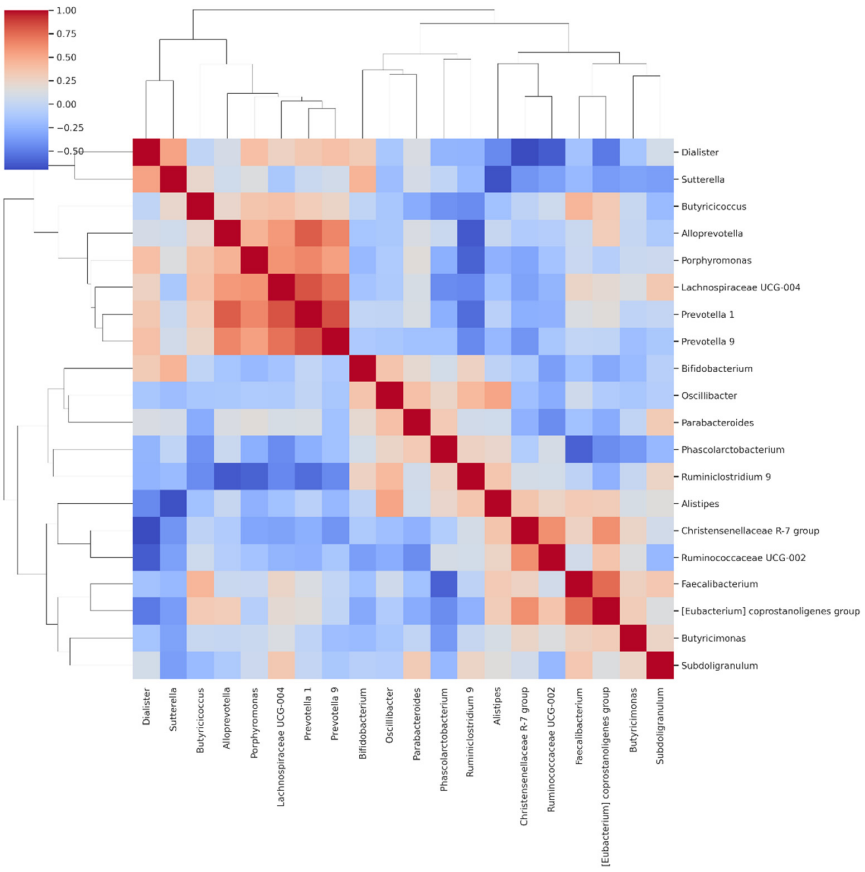

Figure 1. Correlation heatmap of control subjects.

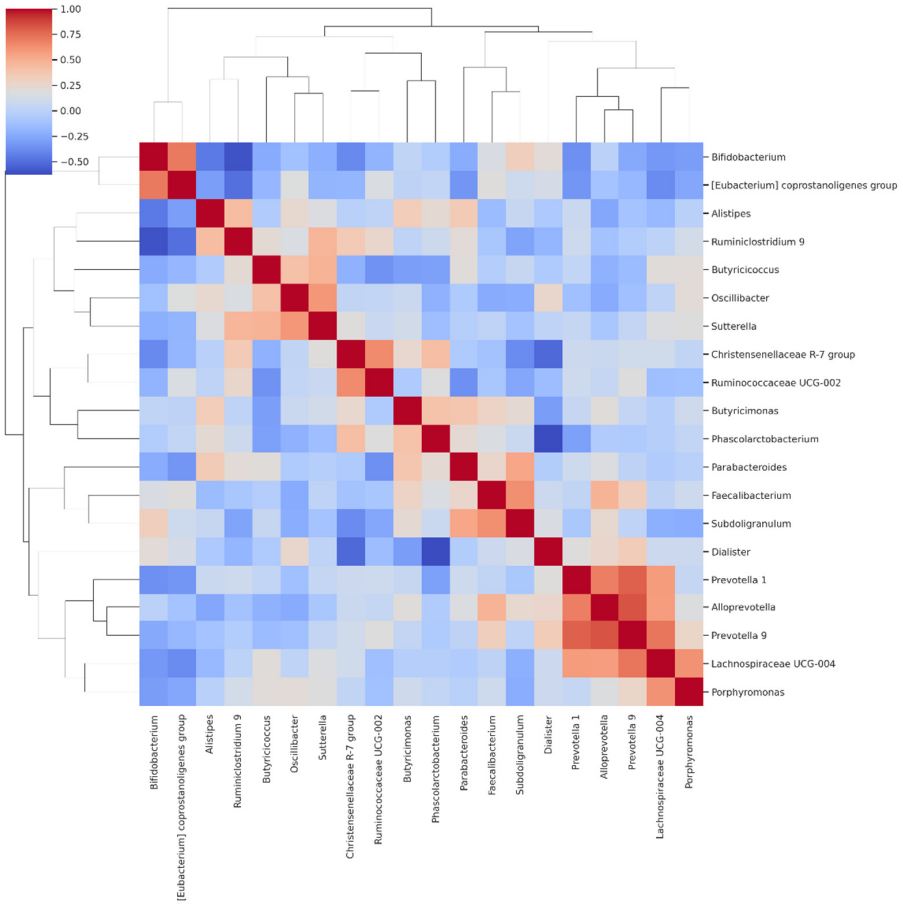

Figure 2. Correlation heatmap of patients with OSA.

There was no difference in Shannon indices, either at phylum (p = 0.35) or genus (p = 0.77) level.

As a next step, we investigated similarities or dissimilarities between the two groups based on the CLR data. Using PERMANOVA we did not observe any significant results. To further investigate, we plotted beta-diversity heatmaps at the four examined taxonomic levels using the subset of microbes we obtained from the LASSO. Clustering OSA and non-OSA groups using the Euclidian norm as a distance measure did not separate them clearly at any taxonomic level. For reference, the beta-diversity heatmap at phylum level clustered on both axis is attached (Figure 3). To visualise if there were any differences between the relative abundances of the selected microbes in patients with OSA and non-OSA controls, we also plotted the same heatmaps with separating the two groups (OSA, non-OSA) on the x axis, which made it possible to point out any differences in the relative abundance of the microbes at different taxonomic levels. With this method, we can see minor differences at the phylum level in the relative abundance of Actinobacteria. CLR values of Actinobacteria are lower in most OSA patients (Figure 4).

3.3. Comparison of Relative Abundances of Bacteria between the OSA and Control Groups

Since we could only reveal minor structural differences between the OSA and non-OSA groups, we continued our analysis by comparing the relative abundance of individual microbes between the two groups. A comparison was achieved using multivariate logistic regression, including potential confounders in the regression, to control their effect on the results.

At the phylum level, the relative abundance of *Actinobacteria* was lower (p = 0.03), whilst the relative abundance of *Proteobacteria* was higher (p = 0.03, Figure 5).

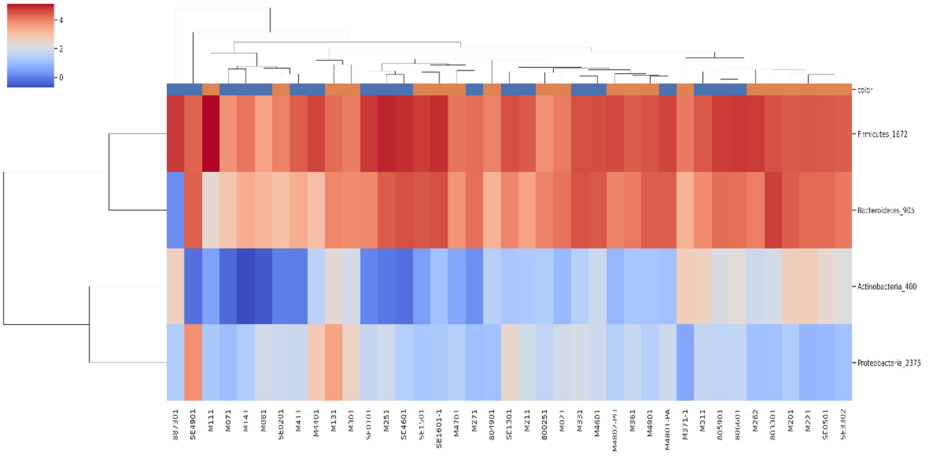

Figure 3. Beta-diversity heatmap at the phylum level. On the top of the figure at the *x* axis, orange colour signals non-OSA controls, and blue colour signals patients with OSA.

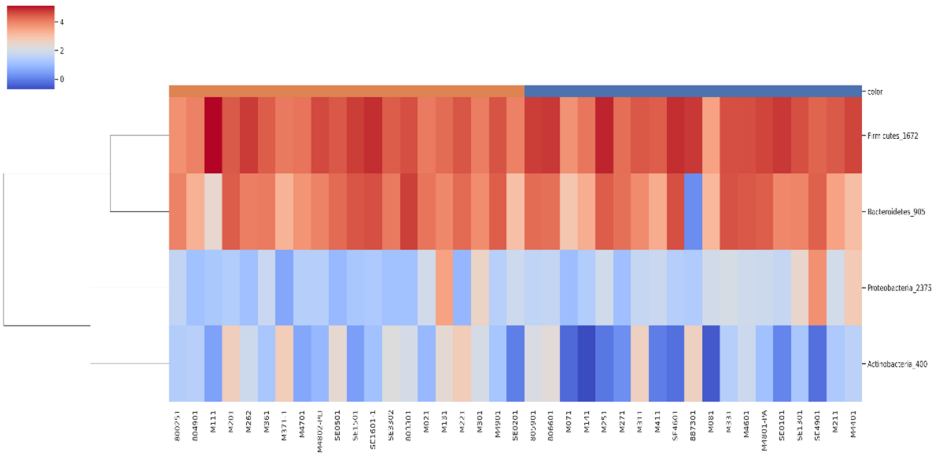

Figure 4. Beta-diversity heatmap at the phylum level. On the top of the figure at the *x* axis, orange colour signals non-OSA controls, and blue colour signals patients with OSA.

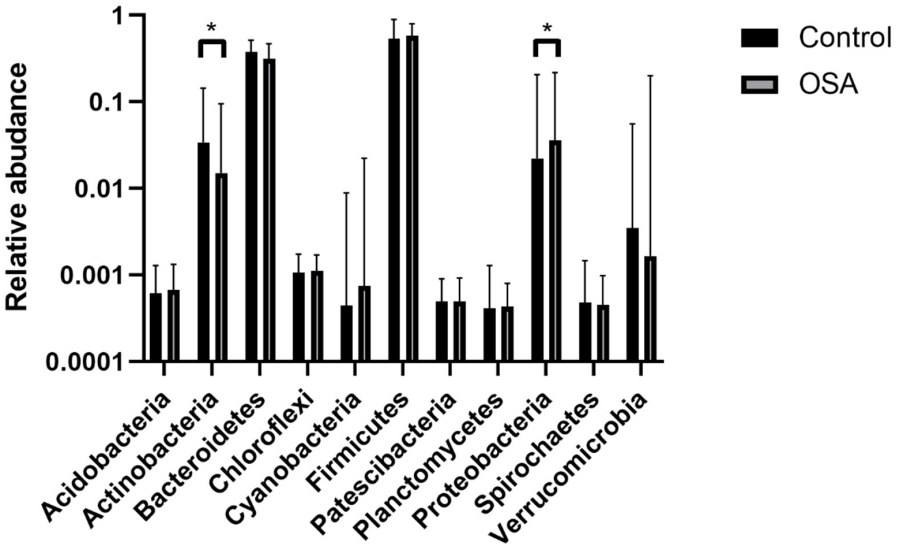

Figure 5. Relative abundance of gut bacteria at the phylum level. Relative abundance of gut bacteria is plotted as median and range. Significant differences between the control and obstructive sleep apnoea (OSA) groups are marked with *.

At the class level, the relative abundance of *Gammaproteobacteria* was higher ($p = 0.01$); at the family level, the relative abundance of *Lactobacillae* was increased ($p = 0.02$) in OSA. At the genus level, the relative abundances of Lactobacillus ($p = 0.02$) and *Roseburia* were higher ($p = 0.03$) in OSA.

Following adjustment for age, gender, BMI, HDL-C, and triglycerides, the difference between the OSA and control groups for *Proteobacteria* ($p = 0.75$), *Gammaproteobacteria* ($p = 0.99$), *Lactobacillae* ($p = 0.87$), and Lactobacillus ($p = 0.83$) became insignificant. The abundance of *Actinobacteria* was still lower ($p = 0.04$), and there was a tendency for higher *Rosaburia* abundances in OSA ($p = 0.054$).

3.4. Correlation between Gut Microbiome and Demographics, Clinical Data and Markers of Disease Severity

There was a significant indirect correlation between the TST90% and relative abundance of *Actinobacteria* ($\rho = -0.34$, $p = 0.04$). The abundance of *Proteobacteria* was significantly related to age ($\rho = 0.38$, $p = 0.02$), BMI ($\rho = 0.39$, $p = 0.01$), HDL-C ($\rho = -0.53$, $p < 0.01$), triglycerides ($\rho = 0.46$, $p < 0.01$), AHI ($\rho = 0.33$, $p = 0.04$), ODI ($\rho = 0.43$, $p < 0.01$), and TST90% ($\rho = 0.43$, $p < 0.01$). The abundance of *Gammaproteobacteria* was related to BMI ($\rho = 0.46$, $p < 0.01$), CRP ($\rho = 0.40$, $p = 0.03$), HDL-C ($\rho = -0.54$, $p < 0.01$), triglycerides ($\rho = 0.47$, $p < 0.01$), AHI ($\rho = 0.36$, $p = 0.03$), ODI ($\rho = 0.47$, $p < 0.01$), and TST90% ($\rho = 0.41$, $p = 0.01$). There was a significant relationship between the abundance of *Lactobacillae* and age ($\rho = 0.36$, $p = 0.03$), BMI ($\rho = 0.39$, $p = 0.01$), HDL-C ($\rho = -0.38$, $p = 0.02$), triglycerides ($\rho = 0.38$, $p = 0.02$), AHI ($\rho = 0.42$, $p < 0.01$), ODI ($\rho = 0.31$, $p < 0.01$), and Sleep% ($\rho = 0.42$, $p = 0.03$). The abundance of the *Lactobacillus* genus was related to age ($\rho = 0.37$, $p = 0.02$), BMI ($\rho = 0.39$, $p = 0.01$), HDL-C ($\rho = -0.37$, $p = 0.02$), triglycerides ($\rho = 0.34$, $p = 0.04$), AHI ($\rho = 0.41$, $p < 0.01$), and ODI ($\rho = 0.52$, $p < 0.01$). Finally, *Roseburia* was related to HDL-C ($\rho = -0.36$, $p = 0.03$) and triglycerides ($\rho = 0.34$, $p = 0.04$).

The abundance of the *Lactobacillae* family and *Lactobacillus* genus was higher in males (both $p = 0.04$). The abundance of *Proteobacteria* and *Gammaproteobacteria* was higher in hypertension (both $p < 0.01$). Similarly, the abundances of *Proteobacteria* ($p = 0.02$) and *Gammaproteobacteria* ($p = 0.03$) were higher in cardiovascular disease. The abundances of *Proteobacteria* ($p < 0.01$), *Gammaproteobacteria* ($p = 0.03$), and *Roseburia* ($p = 0.04$) were higher in dyslipidaemia.

Participants who took any medication had higher abundances of *Proteobacteria* ($p = 0.01$) and *Gammaproteobacteria* ($p = 0.03$). In particular, patients who took benzodiazepines ($p = 0.04$), proton-pump inhibitors ($p = 0.02$) and laxatives ($p = 0.02$) had higher diversity at genus level. In addition, subjects who took proton-pump inhibitors had higher abundance of *Proteobacteria* ($p = 0.03$) and *Gammaproteobacteria* ($p = 0.02$), those who took antihistamines had higher abundance of *Proteobacteria* ($p = 0.04$) and *Gammaproteobacteria* ($p = 0.04$), subjects on clopidogrel had lower abundance of *Proteobacteria* ($p = 0.02$) and *Gammaproteobacteria* ($p = 0.04$), and participants taking laxatives had higher abundance of the *Lactobacillae* family ($p = 0.03$) and *Lactobacillus* genus ($p = 0.03$).

There was no relationship between the abundance of investigated bacteria and smoking status, diabetes, arrhythmia, blood glucose, total cholesterol, LDL-cholesterol levels, TST, SPT, or ESS (all $p > 0.05$).

4. Discussion

In this study, we analysed the bacterial composition of stool samples of patients with OSA and healthy controls. The analysis followed a stepwise process. First, we aimed to identify certain groups of bacteria that could be different in patients with OSA compared to controls. In general, the differences were subtle, but we noticed stronger correlations between bacteria within the control group than in patients with OSA. This could highlight that OSA-related processes, either due to direct (i.e., hypoxemia, sleep fragmentation) or indirect (i.e., differences in metabolism and diet) stimuli, affect particular groups of bacteria rather than gut bacteria in general. Second, we performed alpha- and beta-diversity

analyses, which again revealed only minor differences in the composition of gut bacteria between the groups. Nevertheless, the latter analysis did reveal a potential lower abundance of *Actinobacteria* in OSA, which was confirmed in a further direct comparison even following adjustment for significant confounders. Third, we directly compared relative abundances which analysis was adjusted for potential confounders. Although the raw analysis revealed significant differences for a number of bacteria, most of them disappeared following adjustment, except for *Actinobacteria*.

We found a lower abundance of *Actinobacteria* in OSA, and this reduction was related to the degree of overnight hypoxaemia. The latter is in line with a previous report in mice that the gut levels of *Actinobacteria* were directly related to oxygen concentrations [4]. In the only previous study in adults with OSA, the relative abundance of *Actinobacteria* was similar in patients and controls [11]. In contrast, Smith et al. reported an inverse relationship between *Actinobacteria* and the number of awakenings in healthy subjects [27]. In addition, sleep restriction led to decreased abundance of *Actinobacteria* in mice [28]. *Actinobacteria* are known to produce the sleep-promoting gamma-aminobutyric acid (GABA) [29]; therefore, low *Actinobacteria* could contribute to increased arousals in OSA. Although we did not see any relationship between the relative abundance of *Actinobacteria* and sleep quality indices, the lack of association could be due to the limited number of subjects who had polysomnography as a diagnostic test.

We reported an increased abundance of *Proteobacteria*, including *Gammaproteobacteria* in OSA. Both were related to disease severity and to the prevalence of hypertension, cardiovascular disease, and dyslipidaemia. However, the results need to be interpreted carefully, as the difference between the OSA and control groups disappeared following adjustment. Nevertheless, the results are similar to Ko et al., who found that patients with moderate OSA had a higher abundance of *Gammaproteobacteria* compared to controls [11], and Collado et al., who described similar differences in snoring vs. non-snoring children [9]. In addition, the abundance of *Proteobacteria* was related to sleep fragmentation in children [10]. *Proteobacteria* produce lipopolysaccharide, which could cause systemic inflammation. Confirming this, a significant direct relationship was reported between gut *Proteobacteria*, including *Gammaproteobacteria* and plasma lipopolysaccharide-binding protein in obese subjects [30]. Similarly, in healthy subjects, the richness of *Proteobacteria* was related to interleukin-6 levels [27]. In line with this, we detected a direct relationship between CRP and *Gammaproteobacteria*. Both *Proteobacteria* and *Gammaproteobacteria* were related to BMI. Obesity is a risk factor for OSA [31]; therefore, it is not clear if the association between OSA and these bacteria are independent from obesity. Nevertheless, Cortes-Martin et al. reported that *Gammaproteobacteria* is associated with metabolic syndrome, most particularly hypertension in obese subjects [30] which is in line with our results, namely that a relative abundance of *Proteobacteria* is related to cardiovascular comorbidities. Of note, a high-fat diet can increase *Proteobacteria* populations in the colon [32]. Patients with OSA tend to consume a high-fat diet [7] which could eventually be associated with dyslipidaemia. Supporting this, a significant association between *Proteobacteria* and markers, as well as the prevalence of dyslipidaemia, was found in our study. This is further supported by the fact that following adjustment for metabolic confounders, the association between OSA and *Proteobacteria* became insignificant.

We reported a higher abundance of *Lactobacillae* in the stool samples of patients with OSA. Most of the studies on animal models showed an inverse relationship between *Lactobacillae* and intermittent hypoxaemia [6]. In contrast, a significant relationship between stool *Lactobacillus* and serum homocysteine levels were found in patients with OSA, suggesting that *Lactobacillus* may play a role in the development of cardiovascular diseases [11]. *Lactobacillae* could also produce GABA [29] and sleep fragmentation could lead to decreased *Lactobacillae* [5]. In line with this, we found a significant relationship between *Lactobacillae* and sleep efficiency. Gut *Lactobacillae* are related to obesity and metabolic disease, such as diabetes and dyslipidaemia [33]. Similarly, we found a relationship between *Lactobacillae*, BMI, and deranged lipid parameters. Taking into account that animal models suggest

that intermittent hypoxaemia and sleep fragmentation lead to lower *Lactobacillae* levels, we hypothesise that the observed direct relationship between *Lactobacillae* and OSA in the current study is significantly driven by obesity rather than OSA. Further studies comparing obese and non-obese patients with OSA are warranted in order to explore this relationship in detail. Of note, the association between *Lactobacillae* and OSA became insignificant following adjustment for metabolic confounders. Similarly to the results on *Proteobacteria*, this suggests that metabolic associations (i.e., diet) and potential consequences (i.e., diabetes and dyslipidaemia) drive the relationship, rather than intermittent hypoxaemia.

Finally, we found a higher abundance of *Roseburia* in OSA; interestingly, however, the abundance was not related to disease severity but to dyslipidaemia. *Roseburia* is strongly associated with a protein-free, high-carbohydrate diet [8], which is linked to OSA [7]. Therefore, we believe our findings reflect dietary differences between patients and controls more than OSA-related factors per se.

Taking medications may affect the gut microbiome [34]. Although there was no difference between the OSA and control groups for most medications (except ACE inhibitors), some medications indeed affected the gut microbiome in our study. Of note, there was no difference in the prevalence of subjects taking benzodiazepines, proton-pump inhibitors, antihistamines, laxatives, or clopidogrel; therefore, the observed differences were unlikely due to different medication habits. Further studies need consider the potential effect of medications when interpreting gut microbiome data.

Our study has limitations. First, although the sample size was comparable to most human studies [6], some differences may have not been significant due to type II error. Second, in general, the disease severity in the OSA group was relatively mild, and analysing patients with a broader range of severity could reveal further associations between AHI and the microbiome. Third, this was a cross-sectional study, and the causality between OSA, gut microbiome, metabolism, and cardiovascular consequences cannot be investigated. Forth, some subjects had cardiorespiratory polygraphy as a diagnostic test; therefore, indices of sleep quality were estimated in a limited number of subjects. In addition, the AHI in subjects who had polygraphy may be underestimated, as hypopnoeas related to arousals were not counted. Finally, the diet was not controlled in the current study; therefore, some of the results might have been driven by differences in diet rather than OSA. Further studies investigating a larger and balanced group of subjects could address these limitations. We believe that our results will serve as a basis for these studies.

In summary, we found only very small differences in the composition of the gut microbiome in patients with OSA compared to non-OSA controls. Most particularly, the relative abundance of *Actinobacteria* was lower in OSA confirmed in different models. Although other bacteria, such as *Proteobacteria* and *Lactobacillae*, were also different in OSA, these alterations were most likely driven by metabolic processes associated with OSA than OSA itself. Nevertheless, they might still be relevant in the development of cardiovascular diseases associated with OSA and could serve as a potential target for treatment development. We believe our results serve as a basis for further large-scale studies in order to understand the role of the gut microbiome in OSA.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app12042007/s1, Table S1: The LASSO analysis of the selected species.

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Data Availability Statement: Data supporting reported results can be found by the authors at request.

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