

Correlations of Gut Microbiome, Serum Metabolome and Immune Factors in Insomnia

Honghao Man

Shandong University

Bin Chen

Shandong University

Qinghua Wang

Qilu Normal University

Shujie Fu

Weihai Central Hospital Affiliated to Qingdao

Guangning Xie

Shandong Institute of Industrial Technology for Health Science and Precision Medicine

Jingwen Wang

Shandong Normal University

Changying Zhao

Qilu Children's Hospital

Zhongtao Gai

Qilu Children's Hospital

Chunling Zhang

The Affiliated Central Hospital of Qingdao University

Xueyuan Heng

Linyi People's Hospital

Shengnan Huo

Shandong Institutes for Food and Drug Control

Jianfeng Wang

Shandong Institute of Industrial Technology for Health Science and Precision Medicine

Kyle B. Stirling

Indiana University

Junjie Yang

Qilu

Lei Zhang (✉ microbiome@foxmail.com)

Shandong Human Microbiome Initiative <https://orcid.org/0000-0003-4284-2604>

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Abstract

Insomnia is a common sleep disorder of unclear etiology characterized by individuals experiencing the inability to sleep or the difficulty staying asleep. Gut-brain interaction is being explored with the intent of discerning gut microbiota and its role in many brain-related conditions including insomnia. The number and diversity of gut microbiota that colonize the digestive tract could have a significant association with insomnia, given the microbes that colonize the digestive tract integrate with and impact on the central nervous system, the immune system and multiple metabolic pathways. We aim to examine the diversity and to explore the functional impact of gut microbiota in insomniacs by examining fecal microbiome using 16S rRNA gene sequencing, serum metabolome using ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS), and various immune factors. Our results discover altered and distinct gut microbiota in insomniacs, with enriched *Desulfovibrio*, *Lactobacillus* and *Streptococcus*, and decreased abundance of *Bifidobacterium*, *Gardnerella*, *Sneathia*, *Aerococcus* and *Atopobium*. Non-targeted metabolomics identify 31 aberrant metabolites and implicate metabolic pathways in insomniacs. Most importantly, correlations across gut microbiome, serum metabolome and inflammatory factors are unraveled. Our study provides a better understanding of gut microbiota's role in insomnia and new insights into potential novel etiologies for insomnia.

Introduction

Insomnia is a widespread human problem and a disorder where patients have difficulties initiating or maintaining sleep and typically experience non-restorative sleep for at least three nights per week over a 3-month period¹. It is a multifactorial condition that appears to have association, comorbidity, or cooccurrence with various diseases including diabetes, cardiovascular conditions, and obesity². Although the pathogenesis of insomnia is still unclear, it appears that behavioral, cognitive and emotional factors can also play a role in the occurrence and development of insomnia. Current neurobiological and psychological viewpoints indicate that changes in brain function and genetics can also play a role. Whereas the magnitude and pathophysiological impact of insomnia disorder has been investigated in the central nervous system and immune system^{3,4}, less attention has been paid to the potential interaction effect between the insomnia condition and the potential microbial dysbiosis of gut microbiota. The gut microbiota is involved in the regulation of the phenotypic characteristics of the host⁵, such as, immunity, metabolism⁶, and circadian clock function⁷. Moreover, many complex disorders are now considered to be related to microbiota where previously these disorders were attributed to lifestyle factors⁸⁻¹⁰. Sleep disorder could also impact the metabolism of multiple substances in the human body. We hypothesized that disrupted patterns of sleep may be related to the metabolite abnormality via the changes in population of gut microbial communities. To further explore and illustrate our hypothesis, we analyzed the intestinal microbiome and metabolome of the insomniacs. We posited the non-targeted metabolomics analysis could potentially disclose the aberrant metabolites and metabolic pathways in insomniacs. The combined analysis of both metabolic groups and intestinal microbial distribution will explain some of the relationships between the gut microbiota and host metabolic phenotypes. We

decided it is important to assess the gut microbiota related metabolic phenotype alterations in order to further understand the potential pathophysiological mechanism of insomnia and explore the role of gut microbiota in the development of insomnia.

In this study, the fecal and serum samples were collected from the insomniac patients and the control participants of good sleep history to explore and document the potential role of gut microbiota and the underlying metabolic mechanisms in insomnia. With this purpose in mind, we performed 16S rRNA gene sequencing for fecal and UHPLC-MS/MS analysis for serum. Meanwhile, the immune factors IL-1 β , IL-6 and TNF- α were also measured using Quantitative Enzyme-linked Immunosorbent Assay (ELISA). The serum level of TNF- α and IL-1 β were observed to be significantly different in the insomniacs. The serum metabolic parameters were evaluated by the UHPLC-MS/MS analysis and 31 potential biomarkers were screened out from the patient observations. Our results provided new insights and additional data points for the exploration of new potential mechanisms and origins of insomnia.

Material And Methods

Study design and participants

This study was approved by the Institutional Review Boards of the Weihai Central Hospital (IRB# WCH2017-1201). Informed consent was obtained from all participants. Procedures were carried out in this study to conform with the ethical standards stipulated by the institutional and national research committee, as well as those stipulated in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Our study recruited 40 insomnia patients and 40 healthy controls of good sleep history (Table 1). The Inclusive criteria were as follows: 1) Participants met criteria for primary insomnia defined according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) that included: a. unsatisfied with the time or the quality of sleep, including difficulty in falling asleep, prolonged sleep and early awakening. b. suffering from sleep disorders that cause serious daytime dysfunction or damage (such as emotional or cognitive disorders, work dysfunction). c. insomnia attacks at least three nights a week and sleep disorders occurring for at least 3 months. d. the presence of sleep disorders occurring even though there are adequate sleep opportunities. 2) The healthy volunteers who were recruited from among the people also receiving a physical examination at the same time. These volunteers served as control group participants. 3) Participant age was limited to those 18 years old or above. The exclusion criteria were established as follows: 1) Secondary insomnia caused by somatic or psychiatric diseases. 2) Suffering from diabetes, infectious diseases, gastrointestinal diseases and other diseases. 3) Smokers and drinkers. 4) Those who cannot communicate and cooperate normally. 5) Those with a history of radiotherapy and chemotherapy. 6) Pregnant women. 7) Anyone that had taken antibiotics in the previous three months.

Table 1. Characteristics of participants

	Insomnia	Healthy control
Number	40	40
sexuality♣male♣femal♣	13:27	10:30
Average age♣years♣	59.23±9.35	55.85±9.02
Age distribution♣years♣	40~77	43~74

Sample collection and preparation

The feces samples were collected by the fecal collectors that were pre-loaded with 600-800 mL absolute ethanol¹¹. The samples were immediately frozen in a -80 °C refrigerator. Three samples were collected from each participant. One was used for the extraction of DNA; the other two samples were reserved for the further studies. All the venous blood samples were extracted between 8:30-10:00 AM using vacutainer tubes without anticoagulants (serum), and then the samples were stratified at room temperature for 1 h. The supernatant of the blood was saved after 3,000 rpm centrifugation at room temperature for 1 minute. The serum samples were collected from the supernatant after 12,000 rpm centrifugation at 4 °C for 10 minute and frozen at -80 °C for use.

Microbial community analysis based on 16S rRNA gene sequencing

The total DNA of the gut microbiota was extracted from the fecal sample by utilizing the modified CTAB methods¹². For the purpose of analyzing the taxonomic composition of the bacterial community, the V1-V2 region of the 16S rDNA gene was selected for the subsequent pyrosequencing. The specific primer pair 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 355R (5'-GCTGCCTCCCGTAGGAGT-3') were used for the PCR amplification. The 8 bp Barcode sequence and 5-8 random sequences were introduced into the 5' end of the primers to distinguish samples, remove chimeric sequences and PCR redundancy. The amplification of each sample was performed in triplicate, then products were purified and quantified, and sequenced by Illumina HiSeq 2500 sequencing platform.

The reads were merged by FLASH¹³, the sample split and quality control were executed by the scripts of QIIME (version 1.9.1)¹⁴. The chimeras were removed by using the *de novo*-based method of Usearch6¹⁵. An open Operational Taxonomic Units (OTU) clustering strategy was adopted. The Greengenes database (version 13.8) was served as the reference (the similarity was set to 97%), the unaligned sequences were clustered by UCLUST. The OTU is clustered by *de novo* clustering strategy, and the RDP were used for the

species annotation¹⁶. And the OTUs which abundance less than 0.005% of the total number were filtered. Then, the OTU table was rarefied to a sequencing depth of 10,000 per sample for subsequent analyses of alpha and beta diversity.

Serum metabolites analysis based on UHPLC-MS/MS

The 100 µL serum sample was thawed on the ice, and then 300 µL methanol (containing the internal standard, L-2-chlorophenylalanine 1 ug/mL) was added in to the tube, mixed by a vortex mixer for 30 s; after the ultrasonic treatment in the ice-water bath for 10 min, the protein was precipitated at - 20°C for 1 h; the samples were centrifuged at 4°C for 15 min at 12,000 rpm. For each sample, 2 µL of the supernatant was used for the UHPLC-MS/MS analysis.

We performed chromatographic analysis on a UHPLC system (1290, Agilent Technologies) equipped with a HSS T3 column (2.1 mm × 100 mm, 1.8 µm) coupled to Q Exactive (Orbitrap MS, Thermo). The mobile phase composition was as follows: (1) Positive ion mode: mobile phase A: 0.1% formic acid aqueous solution; mobile phase B: acetonitrile. (2) Anion mode: mobile phase A: 5 mmol/L ammonium acetate aqueous solution; mobile phase B: acetonitrile.

The first and second mass spectrometry data was collected by the Thermo Q Exactive Orbitrap Mass Spectrometer under the Xcalibur software (version: 4.0.27, Thermo). Bombardment energy (NCE mode): 20 eV, 40 eV, 60 eV, scanning rate: 7 Hz.

Detection of the serum immune factors

The levels of the immune factors were measured by human IL-1β/IL-6/TNF-α ELISA kit (ABclonal Technology) according to the procedures supplied by the manufacturer. The absorbance of each sample was measured by Multiplate Reader Ascent (Thermo) at 450 nm and 630 nm, and the amount of proteins in pg/mL calculated on the basis of a standard curve. Each serum was tested in triplicate.

Data analysis and statistical methods

ProteoWizard software was used to convert the original mass spectrometry data into mzML format. The retention time, identify peaks, extract peaks, integrate peaks and align peaks were corrected by XCMS (version 3.2). Material identification was carried out by using OSI-SMMS (version 1.0) software and self-built database. The Log conversion and Par formatting of data were carried out by using SIMCA software (version 14.1). The normalized data were then to perform the principal component analysis (PCA) and orthogonal projections to latent structures-discriminant analysis (OPLS-DA). The differential metabolites were screened by the variable importance in the project (VIP) in the OPLS-DA model¹⁷. The screened

metabolites were then mapped to the KEGG, PubChem and HMDB databases. Metabo Analyst software (<http://www.metaboanalyst.ca/>) was used for metabolic pathway analysis¹⁸.

Data were presented as mean \pm SD. Statistical comparisons and were analyzed by Mann-Whitney U test using the SPSS 22.0 (Chicago, USA), Chi-squared-tests were used to assess differences across genders. $P < 0.05$ was considered statistically significant. The linear discriminant analysis effect size (LEfSe) was performed on the website (<http://huttenhower.sph.harvard.edu/galaxy/>), using default parameters, Alpha values of Kruskal-Wallis (KW) and rank test and Alpha values of Wilcoxon rank sum test were set to < 0.05 , and LDA values were set to > 2.0 .

Results

Distinct gut microbiome observed in insomniac

The fecal microbiota composition profiles were analyzed by the 16S rRNA gene sequencing. A total of 2,480,664 high quality sequence reads were obtained and $31,008.3 \pm 4,463.188$ sequences for each sample. There were some differences in the composition of each sample at the phylum and genus levels. At the phylum level (Figure 1A), the relative abundance of Bacteroidetes in the gut microbiota of insomniacs and healthy controls was $54.60\% \pm 16.65\%$ and $57.93\% \pm 20.21\%$, respectively; followed by Firmicutes and Proteobacteria; Firmicutes accounted for $33.64\% \pm 12.93\%$ in insomniacs and $32.02\% \pm 18.05\%$ in healthy controls, while Proteobacteria accounted for $10.62\% \pm 12.14\%$ and $8.43\% \pm 9.98\%$ in insomnia patients and healthy controls, respectively. At the genus level (Figure 1B), the relative abundance of *Bacteroides* in the gut microbiota of insomniacs and healthy controls was $31.60\% \pm 21.32\%$ and $33.43\% \pm 22.76\%$ respectively, followed by *Prevotella*, the relative abundance of *Bacteroides* was $19.51\% \pm 23.77\%$ and $21.79\% \pm 26.89\%$ in the gut microbiota of the patients and controls, respectively. Venn diagram chart showed the core shared OTU (at least 50% of the samples in each group contain the OTU) in insomniacs and healthy controls. There were 692 core OTUs in healthy controls, 681 core OTUs in insomniacs and 611 total OTUs were founded in both two groups. However, there was no significant difference in alpha diversity between the two groups ($P > 0.05$) (Figure S1). The principal coordinate analysis (PCoA) was used to compare bacterial community patterns between the two groups. The results (Figure 1D) showed that there was a significant difference in community patterns between insomniacs and healthy controls based on the unweighted UniFrac distance (Adonis, $R^2=0.061$, $P = 0.0001$).

Linear discriminant effect size analysis (LEfSe) was performed with the purpose of screening potential gut microbiota biomarkers related to insomnia. A LDA histogram was used to represent the predominant bacteria and the structure of the microbiota in insomniac participants and healthy control participants (Figure 2A). We screened a total of 29 biomarkers, including 2 bacterial phyla, 5 classes, 5 orders, 9 families and 8 genera, by using a Linear Discriminant Analysis (LDA) value greater than 2 as the threshold (Table 2). These results are also illustrated in the cladogram (Figure 2B). As shown in Figure 2B, the abundance of *Desulfovibrio*, *Lactobacillus* and *Streptococcus* were significantly enriched in the gut

microbiome of insomniac participants, whereas *Bifidobacterium*, *Gardnerella*, *Sneathia*, *Aerococcus* and *Atopobium* were more abundant in the healthy control participants.

Table 2. Different taxonomy in LEfSe analysis

OTU	Name	Group	LDA value	P value
Phylum	Actinobacteria	HC	2.88582817	8.22E-09
	Fusobacteria	INS	2.423849947	2.92E-10
Class	Actinobacteria	HC	2.741136487	5.19E-09
	Bacilli	INS	3.207435756	0.001144322
	Coriobacteriia	HC	2.291295405	2.92E-06
	Erysipelotrichi	INS	2.283274477	0.015724472
	Fusobacteriia	INS	2.423849948	2.92E-10
Order	Bifidobacteriales	HC	2.741136487	5.19E-09
	Coriobacteriales	HC	2.291295404	2.92E-06
	Erysipelotrichales	INS	2.283274477	0.015724472
	Fusobacteriales	INS	2.423849947	2.92E-10
	Lactobacillales	INS	3.25087112	0.000965062
Family	Aerococcaceae	HC	2.186764193	5.21E-08
	Bifidobacteriaceae	HC	2.741136487	5.19E-09
	Coriobacteriaceae	HC	2.291295404	2.92E-06
	Erysipelotrichaceae	INS	2.283274477	0.015724472
	Lachnospiraceae	INS	3.59373051	0.004957671
	Lactobacillaceae	INS	2.980629362	0.002075563
	Leptotrichiaceae	HC	2.291818463	2.01E-14
	Streptococcaceae	INS	2.981593472	5.20E-05
	Aerococcaceae	HC	2.186764193	5.21E-08
Genus	Aerococcus	HC	2.186764194	5.21E-08
	Atopobium	HC	2.156748523	1.41E-05
	Bifidobacterium	HC	2.838232315	3.51E-08
	Desulfovibrio	INS	2.403106251	0.000188464
	Gardnerella	HC	2.594522952	6.09E-10
	Lactobacillus	INS	2.980629362	0.002075563

Sneathia	HC	2.291818463	2.01E-14
Streptococcus	INS	2.981593472	5.20E-05

Variation of serum inflammatory factors in insomniacs

During sleep, some unique substances are produced by the human body. We wanted to study these to understand difference between observations in the control group observations obtained from the insomniac group. Certain inflammatory factors have also been shown to regulate and affect the sleep in humans¹⁹. However, inflammation and infection could also alter sleep architecture, whereas a lack of sleep could also impair immune function. Therefore, we measured inflammatory factors in the serum such as interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), and interleukin 6 (IL-6) and these measurements were obtained by using ELISA. As Figure 3 indicates, the IL-1 β was significantly elevated (2.315 \pm 2.202 pg/ml in insomniac group participants VS 0.967 \pm 0.745 pg/ml in control group participants, P \leq 0.0001), meanwhile TNF- α was significantly decreased in the insomniac participants (2.055 \pm 1.619 pg/ml in insomniacs VS 3.234 \pm 1.520 pg/ml in controls, P \leq 0.0001). However, the IL-6 level was consistent between the patients and healthy controls (1.842 \pm 1.396 pg/ml in insomniac vs 1.805 \pm 1.541 pg/ml in controls).

Correlation between serum inflammatory factors and gut microbiota

To reveal the potential correlation between the enterobacteria and the inflammatory factors in the insomniacs, the Pearson correlation analysis was used (Figure 4). We found that 4 OTUs of *Bacteroides*, 2 OTUs of *Bacteroides fragilis*, 1 OTU of *Oscillospira* has a significant positive correlation with IL-1 β . And IL-6 positively correlated with 5 OTUs of *Prevotella copri*, 2 OTUs *Faecalibacterium prausnitzii*, 2 OTUs of *Bacteroides*, and 1 OTU of *Anaerostipes*. At the same time, TNF- α revealed a significant positive correlation with the OTU of *Prevotella copri*, *Parabacteroides*, *Oscillospira*, *Butyrivimonas* and *Bacteroides*.

Significant alteration of metabolomic profiles in insomniacs

Metabolic profiling of the insomniacs and healthy controls were acquired by Ultra performance liquid chromatography-tandem mass spectrometer (UHPLC-MS/MS). 5831 peaks were detected, and 5736 peaks remained after using a relative standard deviation de-noising method in positive ion mode (ES+). And in negative ion mode (ES-), 2612 peaks were detected, and 2588 peaks remained after de-noising. To discriminate the metabolic profiles between insomniacs and healthy control groups, we used principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA). The

results indicated a large separation of metabolome between insomniacs and healthy controls in PCA plots (Figure 5A, B) both in ES+ ($R^2X=0.32$) and ES- ($R^2X=0.317$). Furthermore, individuals in insomniac group were separated from and healthy control group as further evidenced by the OPLS-DA score scatter plots both in ES+ ($R^2X=0.169$, $R^2Y=0.905$, $Q^2=0.707$) and ES- ($R^2X=0.175$, $R^2Y=0.84$, $Q^2=0.532$). We performed a permutation test in order to further validate the OPLS-DA model. After 200 permutations, the R^2 intercept was 0.71 and 0.78 in ES+ and ES- respectively, the Q^2 intercept values were -0.87 and -0.73 in ES+ and ES- respectively (Figure S2A, B). In order to screen for differential metabolites, the first principal component of variable importance in the projection (VIP) was obtained (Figure S2C, D). The VIP values exceeding 1 were first selected as differential metabolites, and the Student's t-test also be used. Then we found 25 metabolites in ES+ and 6 metabolites in ES- (VIP >1 $\&P<0.05$, Table S2). We also visualized it with a heatmap (Figure 5E). We found that, in ES+, 1-palmitoylglycerophosphocholine was identified significantly increased, however, 7-hydroxy-6-methoxy-alpha-pyrufuluran, acinospesigenin A, PS(22:2(13Z,16Z)/20:2(11Z,14Z)) and PA(17:1(9Z)/13:0) were significantly decreased in the insomniacs' serum. Meanwhile, aspartic acid, phenylalanine and phosphatidylcholine lyso 20:4 were identified to be significantly decreased in the insomniacs' serum when under ES-. All differential metabolites were then subjected to the regulatory pathways analysis to discover the metabolic pathways exhibiting high correlations with the metabolites. According to P value and influence value, significant abnormalities were found in five metabolic pathways in insomniacs: glycerophospholipid metabolism ($P < 0.05$), glutathione metabolism ($P < 0.05$), nitrogen metabolism ($P < 0.01$), alanine, aspartate and glutamate metabolism ($P < 0.05$), and aminoacyl-tRNA biosynthesis ($P < 0.05$) (Figure 5F, G).

Correlation between serum metabolome and gut microbiota

To explore the functional relationship of the altered gut microbiota and metabolites in insomniacs, the correlation matrixes based on Pearson's rank correlation coefficient were formulated (Figure 6). Correlations between the varied metabolites and the gut microbiota were identified. The 1-palmitoyl lysophosphatidic acid was positively correlated with *Veillonella dispar*. Phenylalanine was positively correlated with *Streptococcus*. Phosphatidylcholine lyso 20:4 was positively correlated with [*Prevotella*], [*Ruminococcus*] *gnavus*, [*Eubacterium*] *biforme*, *Bacteroides*, *Fusobacterium* and *Streptococcus*. What's more, L-5-oxoproline showed a significant negatively correlation with *Atopobium*. And some short chain polypeptides (Ala-Arg-Arg-Asn, Ala-Trp-Arg-Lys, Asn-Gly-Val, Asp-Phe,Phe-Phe, Val-Phe-Arg) were also negatively correlated with *Atopobium*.

Discussion

Sleep is a complex rhythmic physiological process, considerable evidence showing that the gut microbiome not only affects the immune functions of the host but also regulates sleep through the microbiome-gut-brain axis. Early research shown that gut microbiota exhibits diurnal oscillations in composition and function, it also can control metabolic homeostasis²⁰. At present, research on the effects of the gut microbiome on the pathogenesis of insomnia is incomplete. Liu *et al.* demonstrated

that the composition, diversity and metabolic function of the gut microbiota are significantly changed between insomnia patients and healthy controls²¹. But the assessment of metabolic function is only based on PICRUSt function prediction. Smith et al. also confirmed the association between gut microbiota diversity and sleep physiology²². In addition, they found that positive correlations between total microbiome diversity and IL-6. To our knowledge, our study is the first to comprehensively examine the relationship of gut microbiota and immunity with insomnia. It may lead to a better understanding of the bidirectional communication between the host and the gut microbiome and may produce novel strategies for insomnia treatment and intervention.

The shift in populations of gut microbiota has been associated with various diseases, in this study, we demonstrated that α - and β -diversity of the gut microbiota in insomnia patients is significantly altered, which is similar to the result of Liu *et al.*²¹. We found primarily the beta diversity of the gut microbiota in insomniacs is different, alpha diversity rarely varied. What's more, we found *Desulfovibrio*, *Lactobacillus* and *Streptococcus* were observed as significantly enriched in insomnia group, while *Bifidobacterium*, *Sneathia* and *Atopobium* were significantly decreased. The *Streptococcus spp.* are generally regarded as the producers of D- and L- lactic acid²³. The observation of enriched levels of *Streptococcus* will improve the lactic acid content, further result in a lower the colonic pH²⁴, modify fecal microbiota metabolism, and alter intestinal epithelial barrier function, increasing passive intestinal permeability²⁵. The high rates of colonization with Gram-positive fecal *Streptococcus* was observed to be related to the poor sleep. Meanwhile poorer mood was associated with higher *Lactobacillus*²⁶. The lower *Lactobacillus* level was associated with better subjective sleep quality and vigor ratings in the CFSs who were responded to the antibiotic treatment. The results suggested that the emotional responses and behavior of the human would be influenced by the disordered commensal intestinal organisms²⁷. In addition, high abundance of *Lactobacillus* and *Streptococcus* may be more detrimental to the host²⁶. *Desulfovibrio* are the predominant sulfate-reducing bacterial in the human intestinal. *Desulfovibrio* generate hydrogen sulfide which has a wide range of cytotoxic effects, using the sulfites and sulfates as substrates. Hydrogen sulfide is an effective inhibitor of the oxidation of short-chain fatty acids (SCFAs) in cells, thus completing a vicious cycle of mutually exclusive metabolic interactions between host and bacteria²⁸. The deficiency in SCFAs β -oxidation will affects the sleep quality²⁹. Therefore, may be *Desulfovibrio* was associated to the insomnia be involved in the metabolism of SCFAs.

The level of two important immune factors IL-1 β and TNF- α in the serum were observed significantly changed in the insomniacs, although they showed opposite trends of change. The two factors could act on the brain, and cause decreased appetite, sleep disturbance, and depression³⁰. In addition, the factors might also be involved into the regulation of central receptors or activated the hypothalamic–pituitary–adrenal (HPA) axis lead to the release of stress hormones with similar peripheral effects³¹. IL-1 β and TNF- α are involved in the regulation of slow wave sleep (SWS)^{32,33}. IL-1 β can regulate the release of NE, 5-HT and DA in hypothalamus, and participate in sleep regulation by promoting slow wave sleep³⁴. In the previous studies^{35,36}, IL-1 β was increased across sleep deprivation young man. In addition, prolonged

wakefulness will also up-regulated IL-1 β in the brain³³. We found IL-1 β was also significantly increased in the insomniacs. The previous evidence suggested that TNF- α played an important role in sleep regulation, the sleep fragmentation would be increase TNF- α level and may lead to sleepiness³⁷⁻³⁹. The TNF- α was significantly decreased in the insomniacs, meanwhile exogenous injection of TNF- α into animals could induce sleepiness and elicits excess sleep³³. The results suggested additional TNF- α supplementation may help sleep in insomniacs. Accumulating studies indicated the gut microbiota affects not only gastrointestinal function but also immune system by regulating microbiota-gut-brain axis or HPA axis^{40,41}. In some serious cases, the sleep disorder-related conditions may lead to serious neurasthenia and depression⁴²⁻⁴⁴. Zhu et al.⁴⁵ suggest that TNF- α is involved in the regulation of the uptake of 5-hydroxytryptamine (5-HT). By activating mitogen-activated protein kinase, TNF- α catalyze a series of cascade reactions, which ultimately increase the re-uptake of 5-HT and reduce the concentration of 5-HT, while the decrease of 5-HT concentration will eventually induce anxiety and depression. In the present study, we also found that, the changes of immune factors were correlated with the gut microbiota. Although IL-6 did not change significantly in insomnia patients, we still found a correlation between IL-6 and gut bacteria, which is different from the results of Smith *et al.* *Bacteroides* have been shown to increase significantly in the intestines of patients with insomnia²⁷. It plays an important role in the gut microbiome and can be used as an important biomarker for identifying insomnia patients. *Prevotella* produces endotoxins (lipopolysaccharides) and encodes for superoxide reductase which may favor inflammation⁴⁶.

The metabolomics study suggested, the multiple serum metabolites were different in insomnia group. Many studies have shown that lipids in the blood exhibit circadian rhythm changes^{47,48}. Our research also confirms these changes. In the serum of patients with insomnia, 1-palmitoylglycerophosphatecholine, phosphatidylcholine lyso 20: 4 were significantly decreased, while phosphatidylserine and glycerophosphatidic acid were significantly increased. We also found that phosphatidylcholine lyso 20:4 was positively correlated with *Bacteroides*. A study on difference of gut microbiota in primary insomnia with different traditional Chinese medicine found that *Bacteroides* were enriched in fire due to yin deficiency syndrome group⁴⁹. There is a significant correlation between *Bacteroides* and many metabolites, which indicates that *Bacteroides* may be a very important factor affecting sleep. Threonic acid is an ascorbate catabolic product associated with oxidative stress associated with dehydroascorbic acid. In this study, it was found that threonic acid decreased in serum in patients with insomnia, and that the antioxidant capacity of sleep patients was decreased. The level of phenylalanine in the serum of patients with sleep disorders is significantly reduced. Phenylalanine can be used as a precursor of L-tryptophan. Tryptophan is a very important metabolite in brain-gut axis, and many intestinal bacteria can Affecting the metabolism of tryptophan, tryptophan can reduce the time to fall asleep, so it is often used as a hypnotic drug⁵⁰. Phosphatidylserine (PS) plays an important role in repairing brain damage and improving cognitive ability, improving Alzheimer's disease in the elderly, alleviating tension and depression, physical fatigue and anti-depression⁵¹. The decreased PS level may negatively affect the central nervous system of the insomniacs. Interestingly, differential bacteria in gut

of insomnia patients and healthy controls are correlated with many metabolites. *Streptococcus* positive correlation with phospholipid metabolites (PA, PS, PC). *Atopobium* were negatively correlated with short chain polypeptides. This indicates that the reduction of these metabolites in patients with insomnia is related to these two bacteria, and the specific correlation and mechanism need to be further studied.

Conclusion

In summary, our study found differences in gut microbiota, serum metabolites, and serum immune factors between insomniacs and healthy controls, and shown a novel association between them. Moreover, we identified several specific taxa and metabolites, they may be potential biomarkers related to insomnia. Of course, the mechanism of its occurrence would be studied by more cases to provide evidence in our future work. This will help us develop microbiota-based disease diagnosis, prevention and therapeutic tools.

Declarations

Ethics approval and consent to participate

This study was approved by the Institutional Review Boards of the Weihai Central Hospital (IRB# WCH2017-1201). Informed consent was obtained from all participants.

Consent for publication

Not applicable.

Availability of data and material

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

All authors declare that there is no conflict of interest regarding the publication of this paper.

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Authors' Contributions

L.Z. and J.Y. designed the study. H.M., B.C., Q.W., G.X., J.W., S.H. and J.W. did measurements and data analysis. H.M., S.F., Z.G., C.Z. and X.H. obtained samples and clinical details. L.Z., C.B. and K.S. wrote the manuscript. All authors have read and critically revised the manuscript.

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Figures

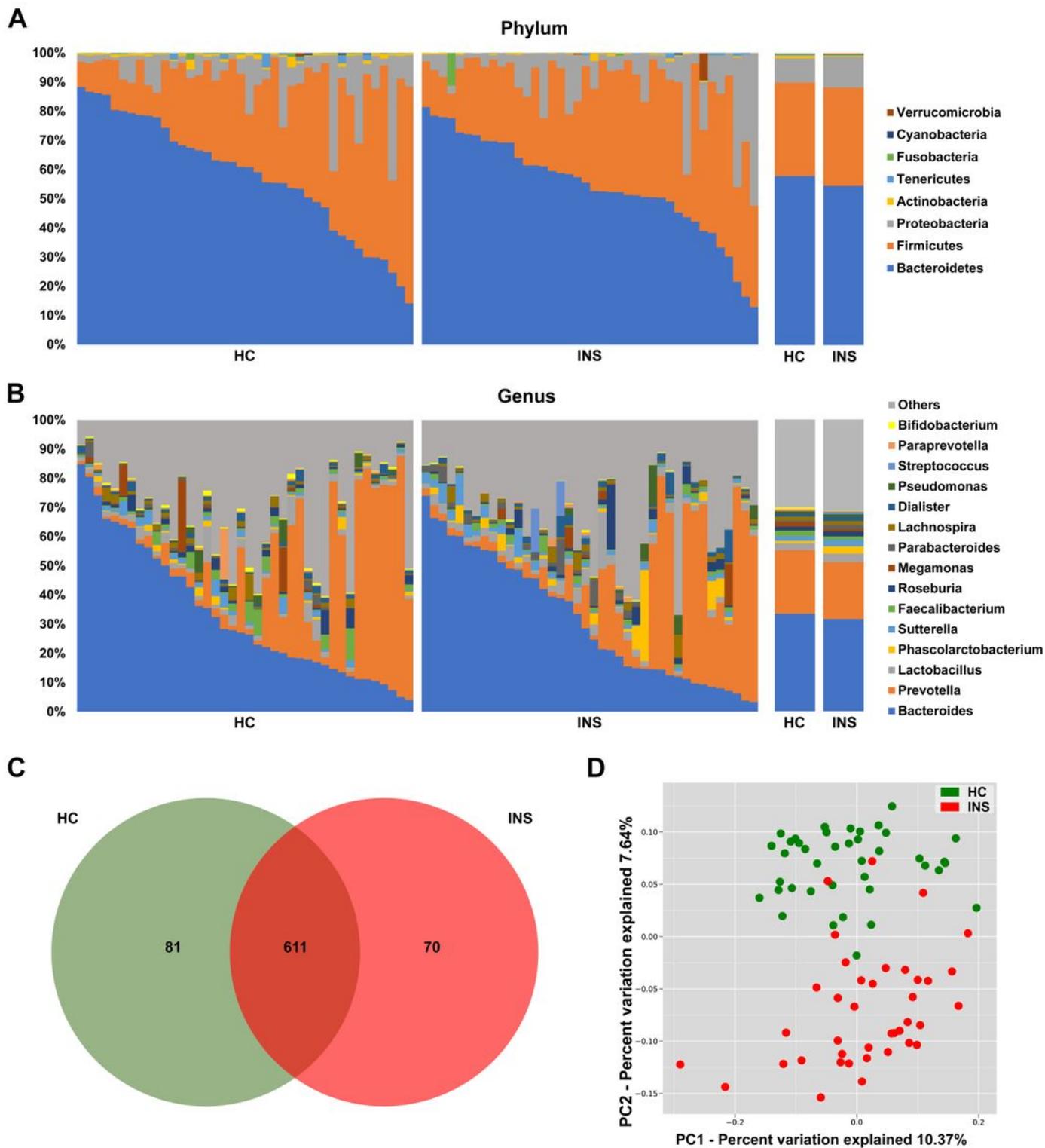


Figure 1

Comparison of gut microbiota between insomniacs and healthy controls. Relative taxa abundance comparison in insomniacs and healthy controls at phylum level (A) and genus level (B). (C) Venn chart of shared core OTUs in insomniac and healthy controls. (D) PCoA analysis based on unweighted UniFrac distance.

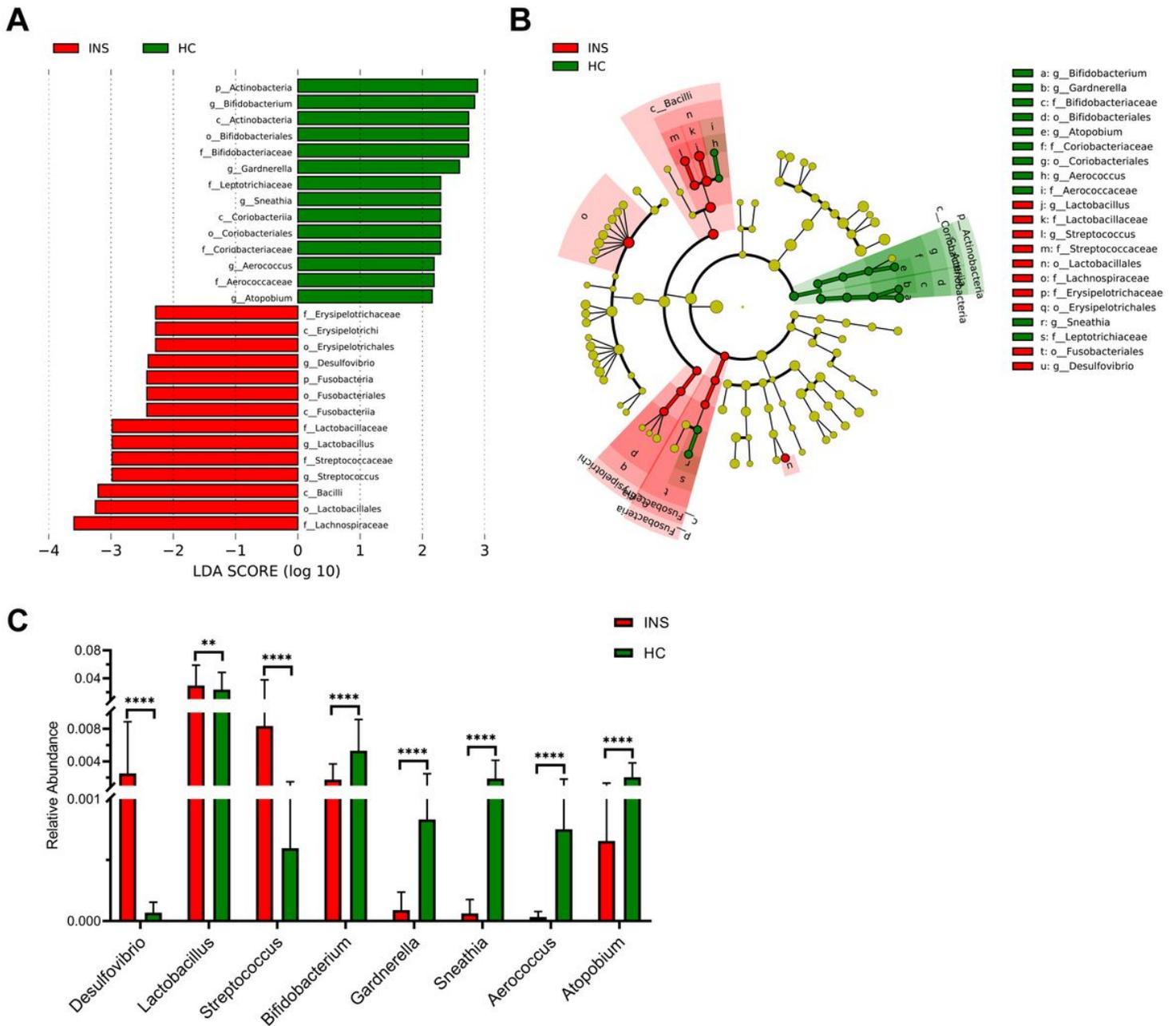


Figure 2

Characteristics of gut microbial community composition in insomniacs and healthy controls. (A) Cladogram representing taxa enriched in gut microbiota community of the insomniacs and healthy controls. (B) Histogram of the LDA scores computed for different abundance levels between insomniacs and healthy controls. (C) The relative abundance of most discriminative genus between insomniacs and healthy controls (** $P < 0.01$, **** $P < 0.0001$).

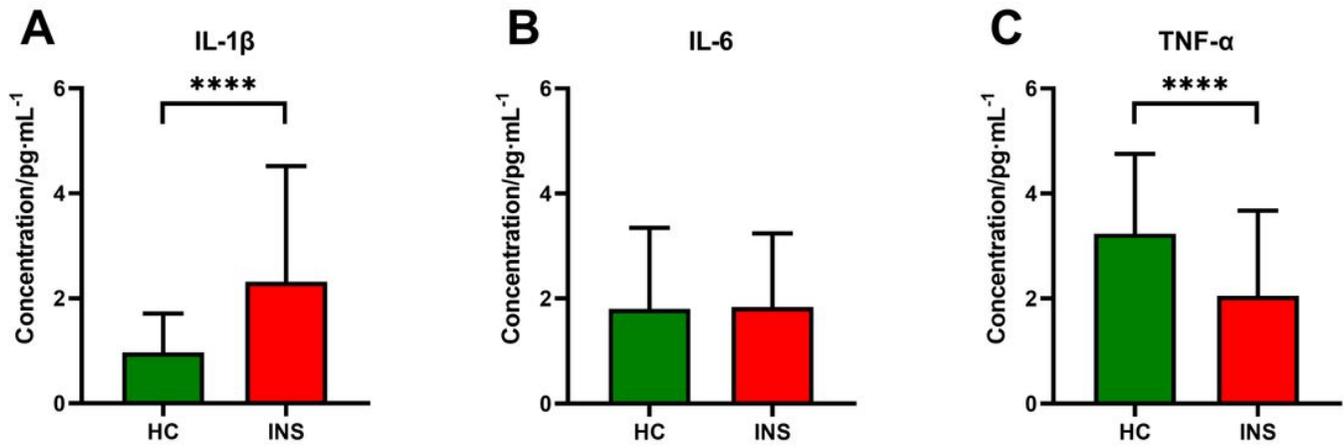


Figure 3

Inflammatory factors level in serum as assessed by ELISA. (A) Histogram of IL- β level between insomniacs and healthy controls. (B) Histogram of IL-6 level between insomniacs and healthy controls. (C) Histogram of TNF- α level between insomniacs and healthy controls. (***) P < 0.001, (****) P < 0.0001)

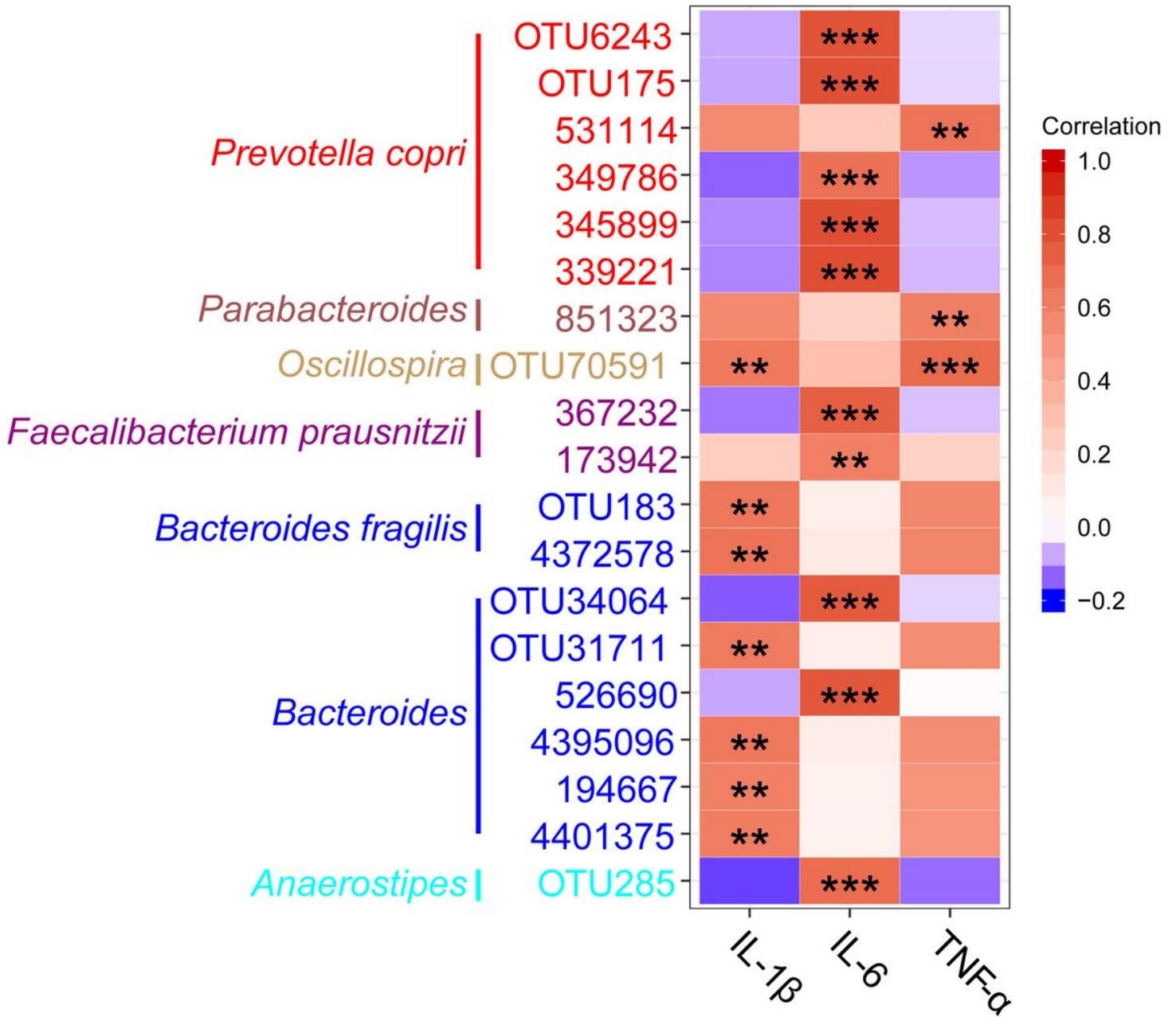


Figure 4

Heatmap of Pearson Correlation Analysis between inflammatory factors and gut microbiota in insomniacs. The heatmap depicts Pearson correlation of inflammatory factors and OTU, assigned to genus and species level, the classifications of OTUs were marked. The R values are represented by gradient colors, where red and blue indicate positive and negative correlations, respectively (** P < 0.01, *** P < 0.001).

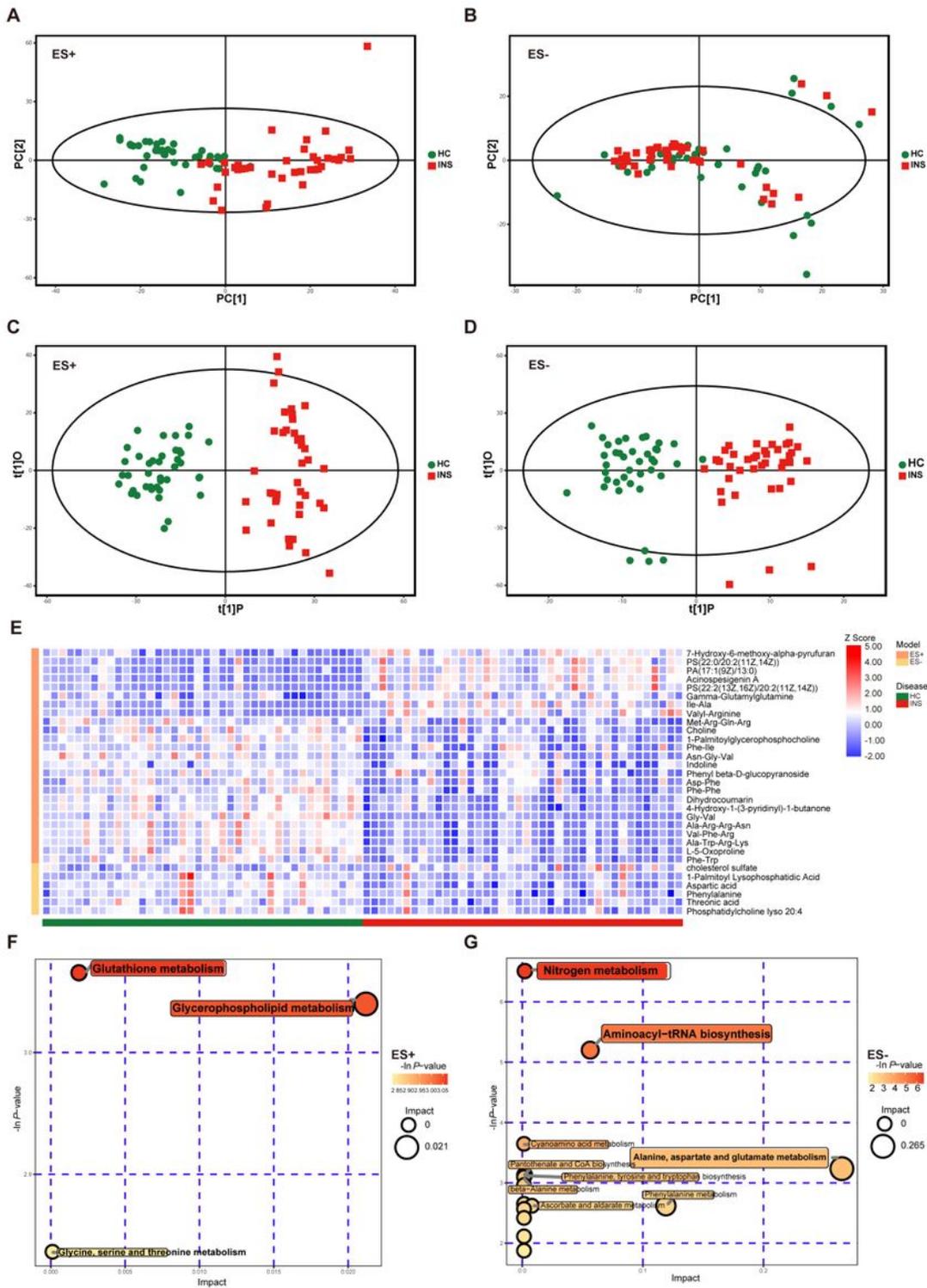


Figure 5

Different metabolites between insomniacs and healthy controls. (A) Score scatter plot of PCA model (ES+). (B) Score scatter plot of PCA model (ES-). (C) Score scatter plot of OPLS-DA model (ES+). (D) Score scatter plot of OPLS-DA model (ES-). (E) The relative amounts of metabolites varied in insomniacs and healthy controls is transformed into Z scores in the heatmap.

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