The mechanism of Peony Seed Oil can promote sleep by 16S rRNA gene sequencing and metabolomics analyses of changes in the intestinal flora and biomarkers in mice

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Abstract:

Peony seed oil (PSO) is an edible oil rich in unsaturated fatty acids. Experimental results show that PSO can be a safe edible oil with sleep-improving effects. We used untargeted metabolomics and 16S rRNA amplicon sequencing to analyze the mechanism by which PSO improves sleep. The results showed that PSO improves sleep by modulating the gut microbiota. Additionally, PSO altered gut metabolites, with some of these metabolites involved in sleep regulation. These results demonstrated that long-term dietary PSO plays beneficial roles in sleep by modulating the gut bacteria and gut metabolism in mice.

Keywords: peony seed oil; Sleep; Metabolomics; Gut microbiota; 16S rRNA

Introduction

Sleep is an extremely important physiological function to maintain human life, which is essential to human body. With the fast pace of today's society and people's working stress increases, 15–35% of adults suffer from regular sleep disruptions, such as difficulties in initiating sleep, insufficient sleep time or frequent waking during the night[1]. Sleep problems is often caused by long-term mental burden, mental work, weak after illness and other causes[2]. Sleep problems will damage people's daytime lives by feeling exhausted and making troubles. Moreover, persistent sleep problems are frequently associated with cardiovascular diseases, obesity, diabetes, and mortality[3].

With the popularization of nutrition knowledge, people's awareness of food, drugs and nutrition has been significantly improved, and more and more attention has been paid to the relationship between diet and health. At present, the research on functional oils is one of the active functional food research fields in the world, which mainly studies the functional role of unsaturated fatty acids[4,5]. It is found that polyunsaturated fatty acids are of great significance in biology and nutrition[6,7].

Recent studies indicate that alterations in the gut microbiota might be associated with sleep through the gut–brain axis[8]. Anderson et al. found that better sleep quality was connected with higher proportions of the gut microbial phyla Verrucomicrobia and Lentisphaerae in healthy adults, suggesting a possible relationship between sleep quality and the gut microbiota[9]. Poroyko et al. showed that chronic sleep fragmentation (SF) was related to the gut microbiota through conventionalization of germ-free mice with the gut microbiota of mice[10].

Peony seed oil contained in the human body needs of polysaccharide, vitamin E, trace elements, and contains a lot of unsaturated fatty acid, which have the potential function of anti-oxidation, auxiliary treatment of diabetes, enhance immunity, anti-inflammation[11]. In China, the current development of peony seed oil is in the stage of vigorous development. The content of unsaturated fatty acids in peony seed oil reached more than 90%[12]. To carry out the study of peony seed oil related active functions for the development of peony seed oil related drugs, health products and high-end edible oil to provide a theoretical basis. At the same time, it is significance to promote national

health and improve economic benefits.

This study aims to investigate the effect of PSO administration on intestinal flora and biomarkers in mice and to explore the potential linkage. To this end, we probed the changes in gut microbiota and intestinal biomarkers after PSO administration and investigated the possible links between them. The differences in gut microbial community structure and gut metabolomics were analyzed by 16S rRNA sequencing technology and LC/MS analysis, respectively. In this work, we (1) compared the composition of peony seed oil with commonly used peanut oil and soybean oil through gas chromatography-mass spectrometry analysis. (2) Established an animal model to observe the regulatory effect of peony seed oil on sleep in mice, and measured blood markers and liver tissue sections to determine the impact of the three oils on the growth process of mice. (3) Analyzed the regulatory effect of peony seed oil on the gut microbiota of mice using 16S rDNA amplicon diversity sequencing. (4) Conducted metabolomics analysis to examine the effects of peony seed oil on mouse feces, and performed integrated omics analysis to reveal the mechanism by which peony seed oil regulates sleep in mice.

2 Materials and Methods

2.1 Determination of unsaturated fatty acids in peony seed oil, peanut oil and soybean oil

This experimental method is based on previous article[13].

2.2 Animal ethics statement

Fifty SPF grade male ICR mice, which were aged 5-6 weeks (30 g). They were purchased from Henan SKobes Biotechnology Co., LTD (Henan, China; License number SCXK2020-0005). The room temperature range was kept at 25 ± 2℃ and the humidity was maintained at 40% ± 5%. In addition, the 12-hour light-dark cycle, the clean bedding, and unrestricted access to both water and standard dry pellet feed. All protocols in this study were approved by the Animal Experiment Committee of Henan University of Science and Technology (No. 20190719016).

2.3 Animal grouping and treatment

After 1 week of feeding, the mice were randomly divided into 4 groups with 5 mice in each group. The groups included the control group (Con group) at a dosage of 0.5 mL normal saline + basal diet. The peony seed oil group (PSO group) at a dosage of 0.5 mL oil + basal diet. The peanut oil group (PNO group) at a dosage of 0.5 mL oil + basal diet, the soybean oil group (SBO group) at a dosage of 0.5 mL oil + basal diet. The basal diet consists of 9% water, 18% to 22% protein, 4% fat, 5% fiber, 8% ash, and 52% to 56% nitrogen-free extract.

2.4 Collection and processing of experimental animal samples

Mouse feces were collected on the last day of the assay for gut microbiome and metabolomics analysis. In addition, the heart was punctured to take blood, serum was taken, and the heart was separated at 3000 rpm for 15 minutes. Livers were collected from sacrificed mice for biochemical analysis. Serum total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) and glucose (Glu) were analyzed by commercial diagnostic kits (Solarbio, China).

2.5 Histological observation of liver tissues

Liver tissues were soaked 1 cm × 1 cm in 4% paraformaldehyde at 4 ℃ for 24 hours, and gradient dehydration was carried out with ethanol of different concentration gradients. Then the liver tissue was treated with xylene to make it transparent. Then the wax was dipped with paraffin wax to control the xylene. The liver tissue after wax immersion was embedded by embedding machine, and bubbles and cracks were avoided as much as possible during the embedding process. Then the slices were sliced and sealed with neutral gum. The sealed slices were placed under a microscope for examination and image acquisition and analysis.

2.6 Sleep assays

2.6.1 Direct sleep test

The mice were given continuous intragastric administration 12 weeks, and the number of sleeping mice in each group was observed after the last oil administration. Sleep is marked by the disappearance of the righting reflex, that is, when the mouse is

placed in the back horizontal position, those who cannot righting for more than 1 min are regarded as asleep.

2.6.2 Prolonged sleep time in mice induced by pentobarbital sodium

Mice in each group were given intraperitoneal injection of pentobarbital sodium at 41mg/kgbw dose (0.1mL/10gbw) 15 min after the last administration of the test substance. The sleep duration of pentobarbital sodium was recorded after the reversal reflex disappeared for more than 1 min as the criterion for sleep.

2.6.3 Sodium barbiturate sleep latency test

Mice were given continuous intragastric administration for 12 weeks, and then intraperitoneally injected barbiturate sodium at the dose of 295 mg/kgbw (0.1mL/10gbw) 15 min after the last administration. The sleep latency time of each group of mice was recorded with the disappearance of righting reflex for 1 min as the index of sleep.

2.6.4 Pentobarbital sodium subthreshold dose hypnosis experiment

Mice were given continuous intragastric administration for 12 weeks, and pentobarbital sodium was intraperitoneally injected into each group at a dose of 32mg/kgbw, the injection amount was 0.1mL/10gbw, and the elimination of righting reflex for more than 1 minute was taken as the sleep index, and whether the mice in each group fell asleep was recorded.

2.7 Gut microbiota analysis

The analysis of the composition of the gut flora involves the extraction of total genomic DNA from the sample. DNA purity was determined on 1% agarose gel and the concentration was adjusted to 1 ng/μL with sterile water. Then, after designing the conserved primers and performing PCR amplification, the sequencing adapter was connected to the end of the primers. Sequencing libraries were prepared with purified PCR products.

The raw readings obtained by sequencing were percolated using Trimmomatic v 0.33 software. Usearch v10 software was used to merge clean reads of each sample, and the length of the combined data was permeated according to the length range corresponding to different regions. The chimeric sequences were detected and removed using

147 UCHIME v4.2 software to obtain the final set of valid reads. Usearch software was
148 used to cluster the sequences, the similarity threshold was 97.0%, and the operation
149 classification unit (OTU) was obtained. SILVA is used as reference database for
150 classification and annotation of feature sequences. QIIME 2 software (v 1.8.0) was used
151 to select the sequences with the most abundant features at the phylum and genus
152 classification level as representative sequences. The α-diversity and β-diversity
153 indices of the samples were evaluated by QIIME 2.

154 **2.8 Untargeted metabolomics analysis of faeces**

155 Fecal metabolites were separated employing a triple TOF-6600 mass spectrometer
156 (AB Sciex, USA) and LC20 ultra-high performance liquid chromatography (UPLC) on
157 a Waters ACQUITY UPLC HSS T3 C18 column (100 mm × 2.1 mm, 1.8 μm,
158 Shimadzu, USA). The column temperature was 40℃ and the sample size was 2 μL.
159 The mobile phase included ultra-pure water in phase A (containing 0.1% formic acid)
160 and acetonitrile in phase B (containing 0.1% formic acid) at a flow rate of 0.4 mL/min.
161 Positive mode: mobile phase A: 0.1% formic acid, mobile phase B: methanol; Negative
162 mode: mobile phase A: 5 mM ammonium acetate, pH 9.0, mobile phase B: methanol.
163 The gradient elution conditions were set as follows: 0 min: 98% A phase, 2% B phase;
164 1.5 min: 98% phase A, 2% phase B; 3 min: 15% phase A, 85% phase B; 10 min: 0% A
165 phase, 100% B phase; 10.1 min: 98% phase A, 2% phase B; 11 min: 98% phase A, 2%
166 phase B; 12 min: 98% phase A, 2% phase B.

167 During LC-MS/MS analysis, positive and negative data are imported into the
168 MetaboAnalyst R package (v 3.1.3). Secondly, partial least squares discriminant
169 analysis (PLS-DA) and orthogonal partial least squares discriminant analysis (OPLS-
170 DA) were used to visualize the metabolic differences among the groups. fold change
171 (FC) method was used to determine the metabolite change amplitude, combined with *P*
172 value, and the metabolites with significant differences between groups were screened.

173 **2.9 Integrative analysis**

174 Spearman correlation analysis was conducted between intestinal metabolites and
175 serum indexes in the top 20 abundance and intestinal microorganisms in the top 20

abundance, and *P*-values were obtained, which were visually displayed using heat maps.

2.10 Statistical analysis

SPSS software v 22.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. One-way ANOVA and least significant difference (LSD) multiple comparison tests were used for comparison between groups. The results were plotted using OriginPro 2024b Learning Edition.

3. Results

3.1 Determination of unsaturated fatty acid composition of PSO, PNO and SBO

We used GC-MS to determine unsaturated fatty acid composition of different oils. They are mainly oleic acid, linoleic acid, linolenic acid. Among them, the content of unsaturated fatty acid in PSO was 91.99% in total (Figure S1, Supplementary Table 1). PNO' unsaturated fatty acid content was 54.02% (Figure S2, Supplementary Table 2). The percentage composition of unsaturated fatty acid of SBO was 63.37% (Figure S3, Supplementary Table 3). These results showed that PSO had the rich unsaturated fatty acid.

3.2 Effects of the long-term intake of PSO on the physiological status of mice.

Hematoxylin and eosin (H&E) staining method was employed to observe liver tissues morphology of mice after dietary PSO for 12 weeks. In Figure 1A-D, we can clearly see that after the three kinds of oil were fed to mice, compared with the Con group, the tissue cells of the liver sections were uniform, closely arranged, with dense nuclei and no fat particles, indicating that there was no difference in food safety between peony seed oil and the other two common edible oils.

Lack of sleep can affect lipid metabolism and lead to elevated blood lipid levels. We used PSO, PNO and SBO to gavage treatment in different group mice. As shown in Figure 2, the level of Glu, TC, TG, HDL-C and LDL-C in PSO group and Con group had little difference, Also, PSO made the level of HDL-C in mice higher. In addition, there was also less difference between the PSO, PNO and SBO groups. These results suggested PSO and other oils for daily consumption have little effect on cholesterol metabolism.

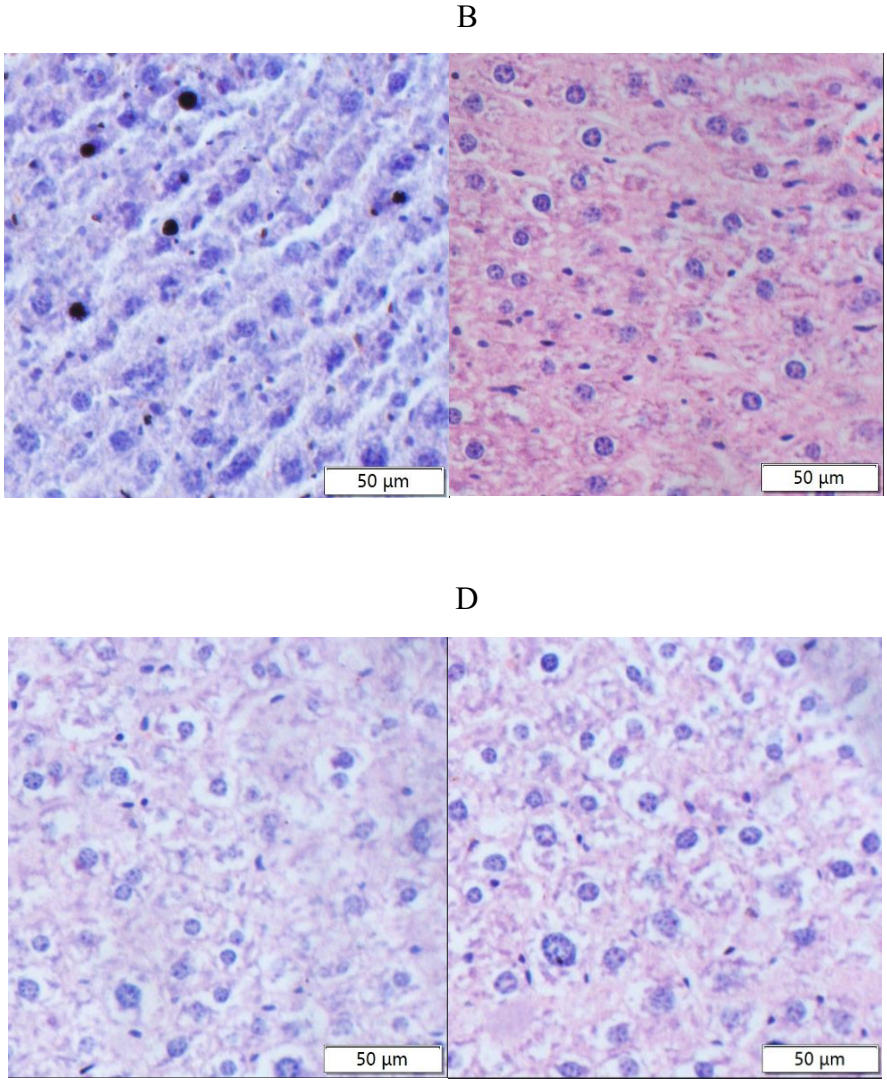

Figure 1. Representative images of hematoxylin and eosin-stained liver tissue for (A) Con group, (B) PSO group, (C) PNO group, (D) SBO group. Scale bar = 50 μm.

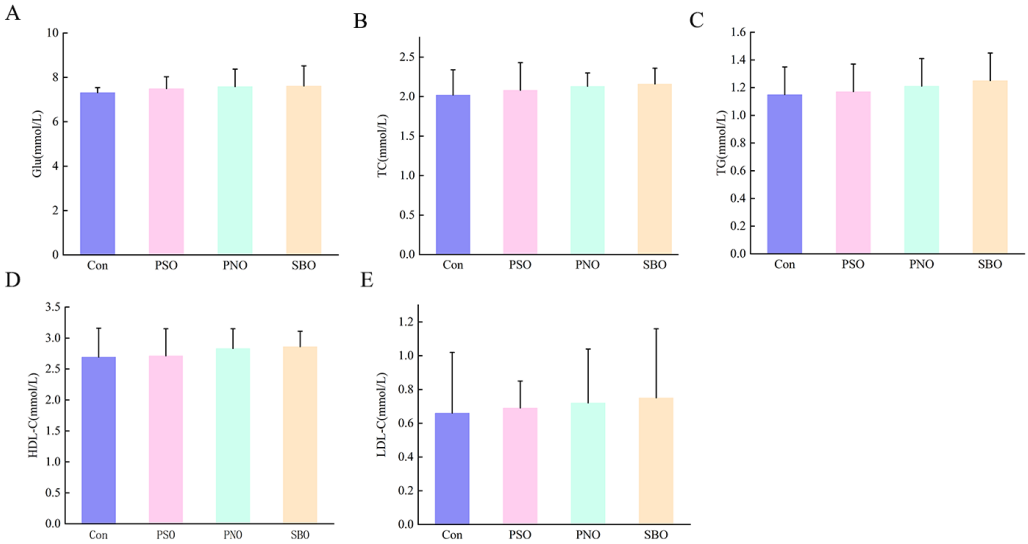

Figure 2 Effect of PSO, PNO and SBO on the levels of Glu (A), TC (B), TG (C), HDL

(D), LDL-C (E) in mice. The data were presented as the mean ± SEM (n = 10).

3.3 Effects of the long-term intake of PSO on improving sleep function in mice

Direct sleep test observation found that mice in the peony seed oil group were more lethargic and had better sleep conditions. Then, the following experiment was conducted to analyze the sleep conditions of mice in different oil groups through data.

As shown in Figure 3A, the sleep time of mice was obviously lengthened in PSO group when compared with the Con group, and there was statistical difference ($P < 0.05$). PNO group and SBO group could prolong the sleep time, but they didn't exist statistical difference. Furthermore, the prolonged rate was 38.4%, 13.3%, 18.6% in PSO group PNO group and SBO group, respectively.

The sleep latency in the PSO group was shorter than that in Con group (Figure 3B), and the differences were significant ($P < 0.05$). However, though PNO and SBO could reduce the sleep latency, but not remarkable. What's more, the reduction rate of sleep latency was 20.1%, 7.4%, 6.9% respectively.

After using pentobarbital sodium to treat mice, the result showed that the rate and the amount of mice in PSO group had great increase compared with Con group (60%). The rates of falling asleep PNO group and SBO group had markedly increase with 30% and 50%, respectively (Table 1).

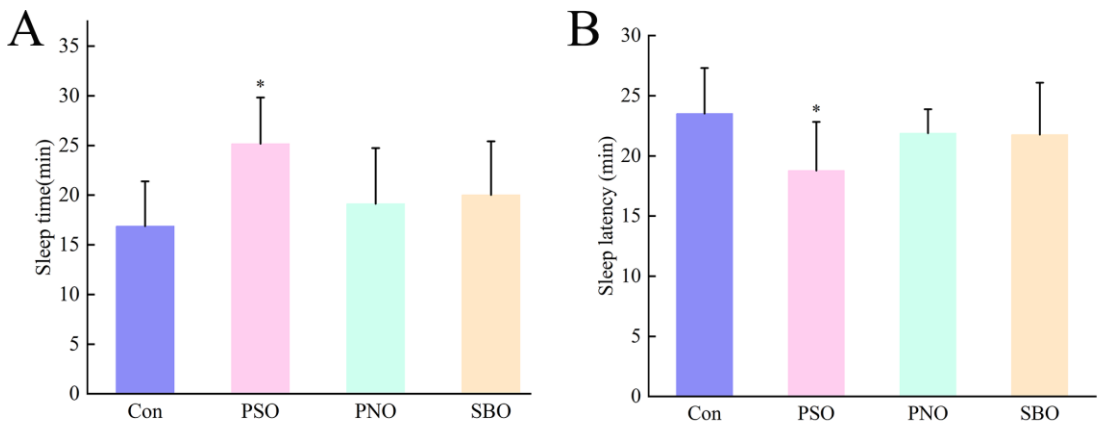

Figure 3 (A) Effect of PSO on the sleep time of mice induced by pentobarbital sodium in sleep improvement experiment. (B) Effect of PSO on the length of sleep latency in

mice induced by barbiturate sodium in sleep improvement experiment. Each bar represents the mean ± SEM (n = 10). *$P < 0.05$ vs Con group.

Table 1 Effect of PSO on the effects of a subthreshold dose of pentobarbital sodium.

Group	Number of sleeping mice	Rate (%)
Con group	2	20
PSO group	6	60
PNO group	3	30
SBO group	5	50

3.4 Effects of the long-term intake of PSO on the gut microbiota

Given that long-term treatment of PSO through diet in direct encounters the intestinal microbial environment, we next tried to delve the regulation of the gut microbiome by PSO. We found 6508 OTUs were found in 24 samples based on 97% sequence similarity (Supplementary Table 4). There are 316 OTUs common to all four groups. There were 1918, 2599, 800, and 875 OUT in Con group, PSO group, PNO group and SBO group, respectively (Figure 4A). From the bar chart (Figure 4B), we could see that the number in PSO group was the most.

3.4.1. Analysis of the alpha and beta diversity of the gut microbiota

Based on alpha diversity analysis, observed features and Chao 1 indexes displayed that the abundance of PSO group was higher than that of Con group, PNO group and SBO group (Figure 5A-B). Moreover, Shannon and Simpson indexes demonstrated there were significant differences in species richness and evenness in PSO group when compared with Con group (Figure 5C-D, $P < 0.05$), but no significant differences between PNO group and SBO group.

Based beta diversity analysis, supervised PLS-DA result showed PSO group and Con had obvious separation. PNO group and SBO group had a cross aggregation. And PSO group had significant separation trend compared to PNO group and SBO group as well (Figure 5E). Principal coordinate analysis (PCoA) of Bray-Curtis distance showed significant separation between peony seed oil group and blank group. In addition, we

observed that the cross-clustering between the peony seed oil group, peanut oil group and soybean oil group was not obvious (Figure 5F). In short, our study demonstrated that PSO altered the abundance and diversity of intestinal flora in mice.

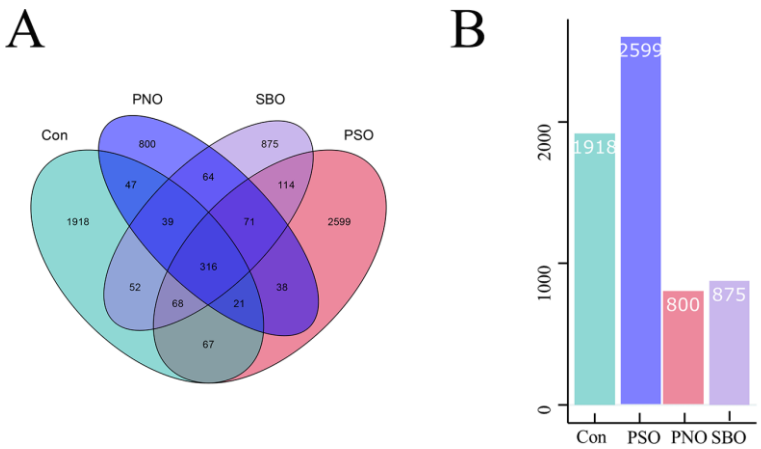

Figure 4 (A) Venn diagram for 4 groups. (B) Bar chart of OTUs for 4 groups.

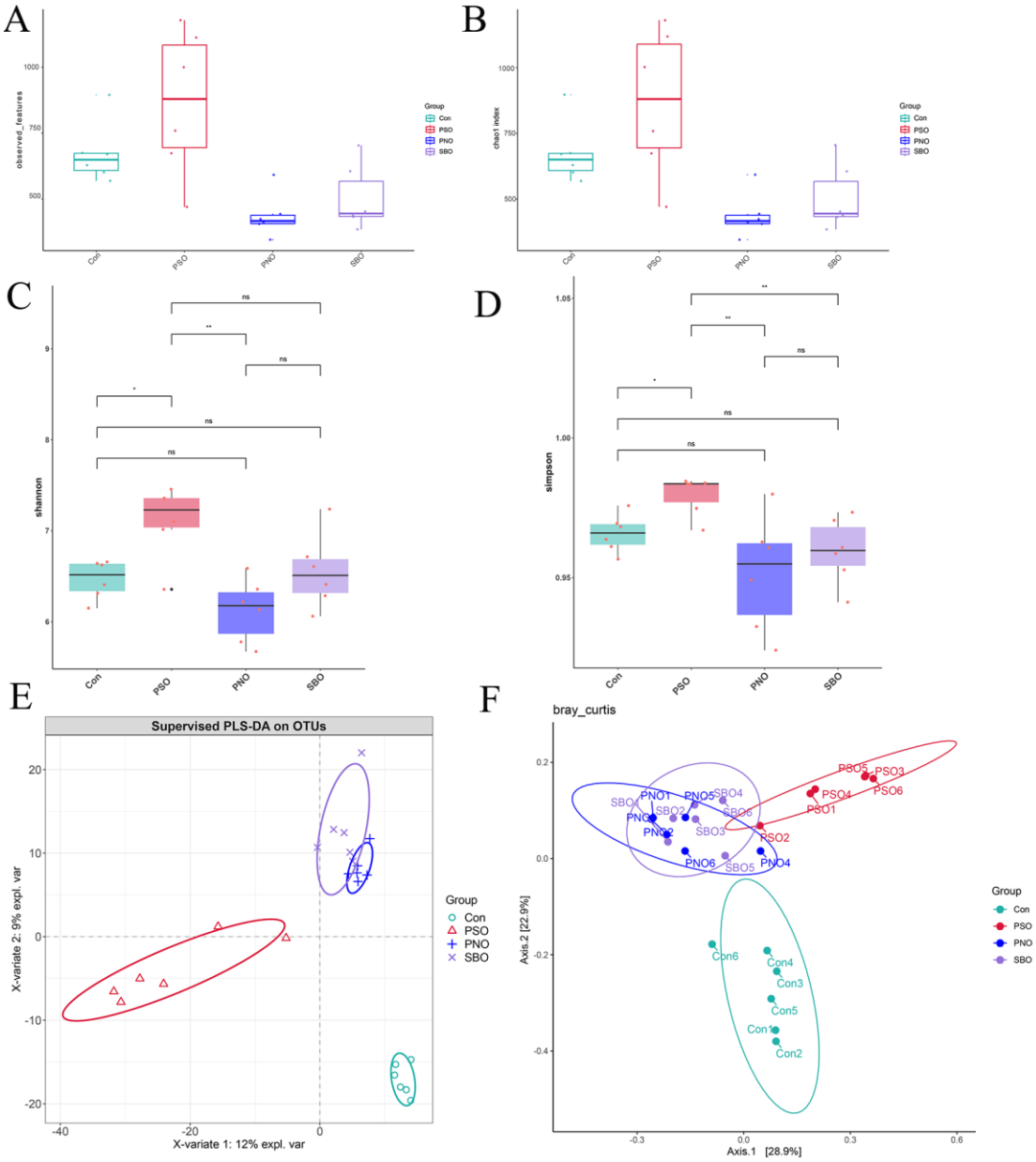

Figure 5 Alpha and beta diversity of gut microbiota among different groups of mice. (A–D) α-diversity indices of the gut microbiota. (A) observed features index, (B) Chao1 index, (C) Shannon index, (D) Simpson's index. (E–F) β-diversity of the gut microbiota. (E) PLS-DA analysis. (F) PCoA analysis of the Bray-Curtis distance based on OTUs. ns: no significant difference, *$P < 0.05$

3.4.2. Analysis of gut microbiota composition

We analyzed the classification and distribution of microbial composition in Con

group, PSO group, PNO group and SBO group. In Figure 6A, At the phylum level, the composition of top 20 gut microbes manifested that Bacteroidetes was the dominant bacteria in the Con group, followed by Firmicutes and Proteobacteria, which accounted for 56.20%. 31.68% and 6.69%. The top 3 predominant bacteria in PSO group are Firmicutes, Bacteroidetes and Proteobacteria, accounting for 44.64%, 44.15% and 6.03% respectively. PNO group was similar to PSO group. The dominant bacteria in the SBO group were Proteobacteria, Firmicutes and Bacteroidetes. At the phylum level, Firmicutes and Bacteroidetes in the treatment group were significantly different from that in the Con group (Figure 6B; $P < 0.01$, $P < 0.05$, $P < 0.001$).

Next, we further analyzed the top 20 taxa at the generic level. As shown in Figure 6C, Lactobacillus, Bacteroides, Prevotella and Prevotella are the dominant bacteria in the Con group, while the dominant bacteria in the PSO group are Lactobacillus. Prevotellaceae_Prevotella, Odoribacter, Allobaculum, etc. The dominant genera in PNO group and SBO group were Lactobacillus, Allobaculum, Bacteroides and Bifidobacterium. In addition, as can be seen from the bar chart in Figure 6D, compared with the Con group, the abundance of Bacteroides, Sutterella, Prevotella and Parabacteroides in the PSO group was markedly reduced ($P < 0.01$, $P < 0.001$). There was no significant difference of PNO group in Sutterella, but the bacteria of Bacteroides, Prevotella and Parabacteroides presented the remarkable difference ($P < 0.05$, $P < 0.001$). Moreover, the abundance of Ruminococcaceae_Ruminococcus, Oscillospira, Coprococcus, Paraprevotella in PSO group had significant increase compared with Con group ($P < 0.01$、$P < 0.05$、$P < 0.001$). However, there was no significant difference of Ruminococcaceae_Ruminococcus, Coprococcus compared to Con group. Also, the abundance of Oscillospira, Coprococcus in PNO group had no significant difference. There findings indicated PSO changed the gut bacterial composition and increased probiotic abundance to regulate sleep.

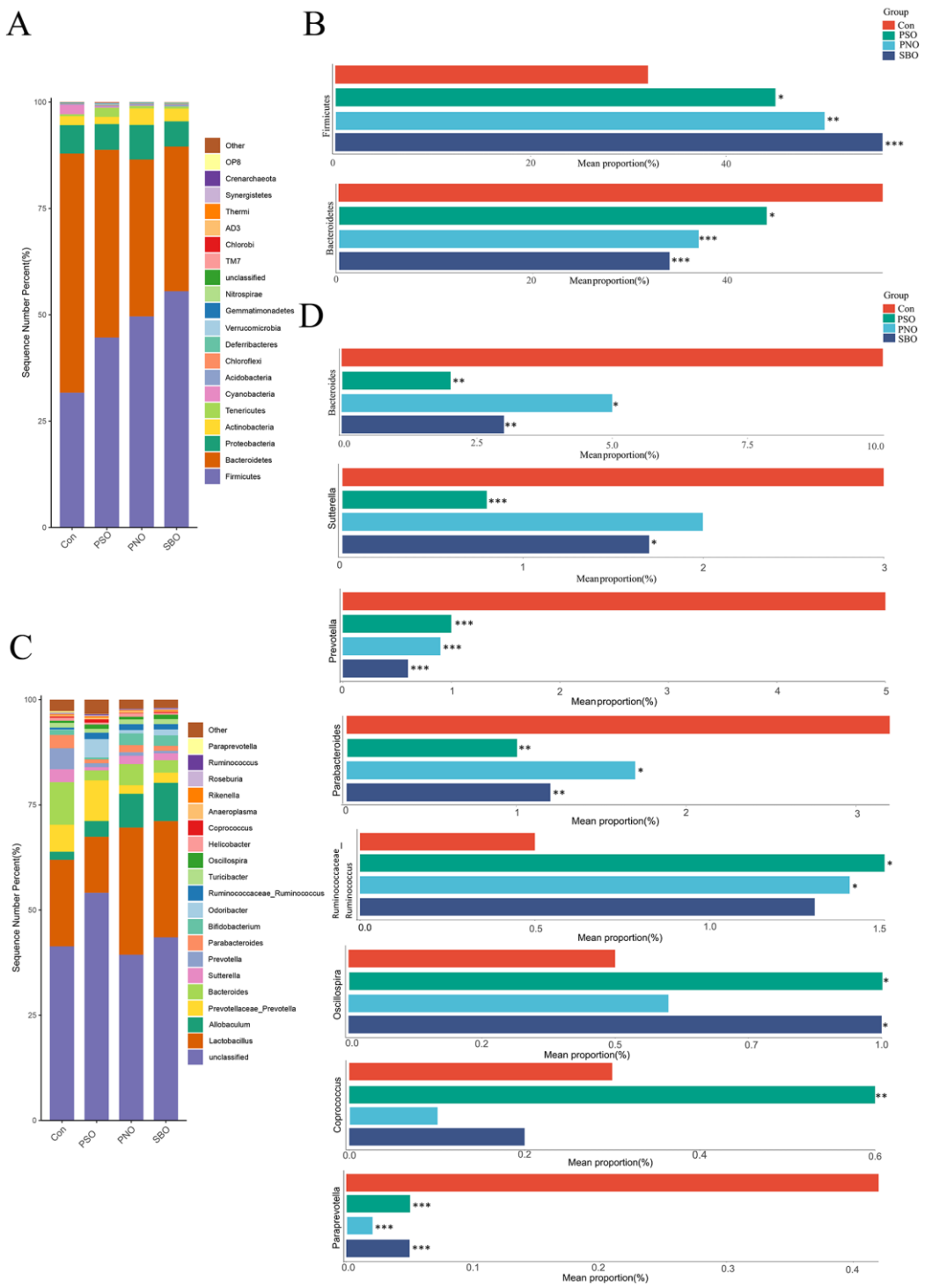

Figure 6 Impact of PSO on gut microbiota composition at the phylum level in Mice. (A) Bar graph illustrating the relative abundance of species at the phylum level (top 20). (B) Species significantly influenced by PSO at the phylum level from the five groups. (C) Bar graph illustrating the relative abundance of species at genus level (top 20). (D) Species significantly influenced by PSO at the genus levels from the five groups. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

3.4.3. Effects of the long-term intake of PSO on fecal microbiota composition and SCFAs

The Linear Discriminant Analysis Effect Size (LEfSe) was used to evaluate the effect of sample abundance on observed differences ($P < 0.05$, LDA > 4.0). The analysis results for Con vs PSO, Con vs PNO and Con vs SBO are presented. As shown in Figure 7, cladogram and LDA scores generated from LEfSe was used to identify biological markers with statistical differences in different groups. The Con group exhibited the enrichment in Bacteroides, Bacteroidaceae, Prevotella, Prevotella, Paraprevotellaceae, Alistipes, Bacteroidales, Bacteroidia, Bacteroidetes. But PSO group was primarily enriched in Prevotella, Odoribacter, Ruminococcus, Prevotellaceae Odoribacteraceae, Clostridia, Clostridiales, Ruminococcaceae, Lachnospiraceae. The PNO exhibited the marker taxa in Bifidobacterium, Bifidobacteriaceae, Bifidobacteriales, Actinobacteria, Actinobacteria. Five taxon differences emerged in SBO groups, including Allobaculum, Erysipelotrichaceae, Erysipelotrichales, Erysipelotrichi, Firmicutes.

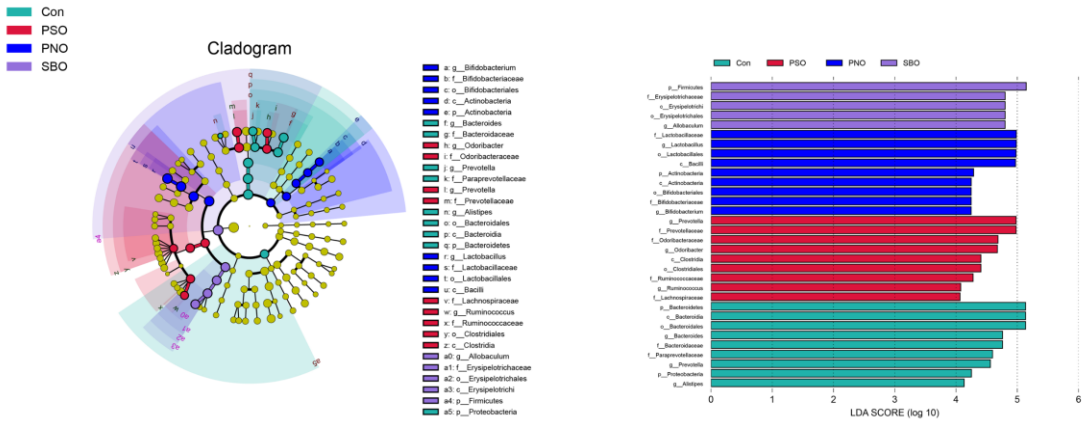

Figure 7 Significantly different taxonomic biomarkers were identified in different groups by LEfSe. The cladogram's circles radiating from the inside out represent the classification level from phylum to genus. Taxa without significant differences are shown in yellow, while taxa with significant differences are colored based on their association with the group exhibiting the highest abundance. Taxa with an LDA score > 4 are marked as statistically significant and listed on the right side of the figure.

Additionally, 2-[5-(2-hydroxypropyl) oxolan 2-yl] propanoic acid is a short-chain

fatty acid (SCFA) and the PSO group displayed higher levels of it in box plot compared to other 3 groups after 12 weeks of administration (top 25 with lower P values, Figure. 8).

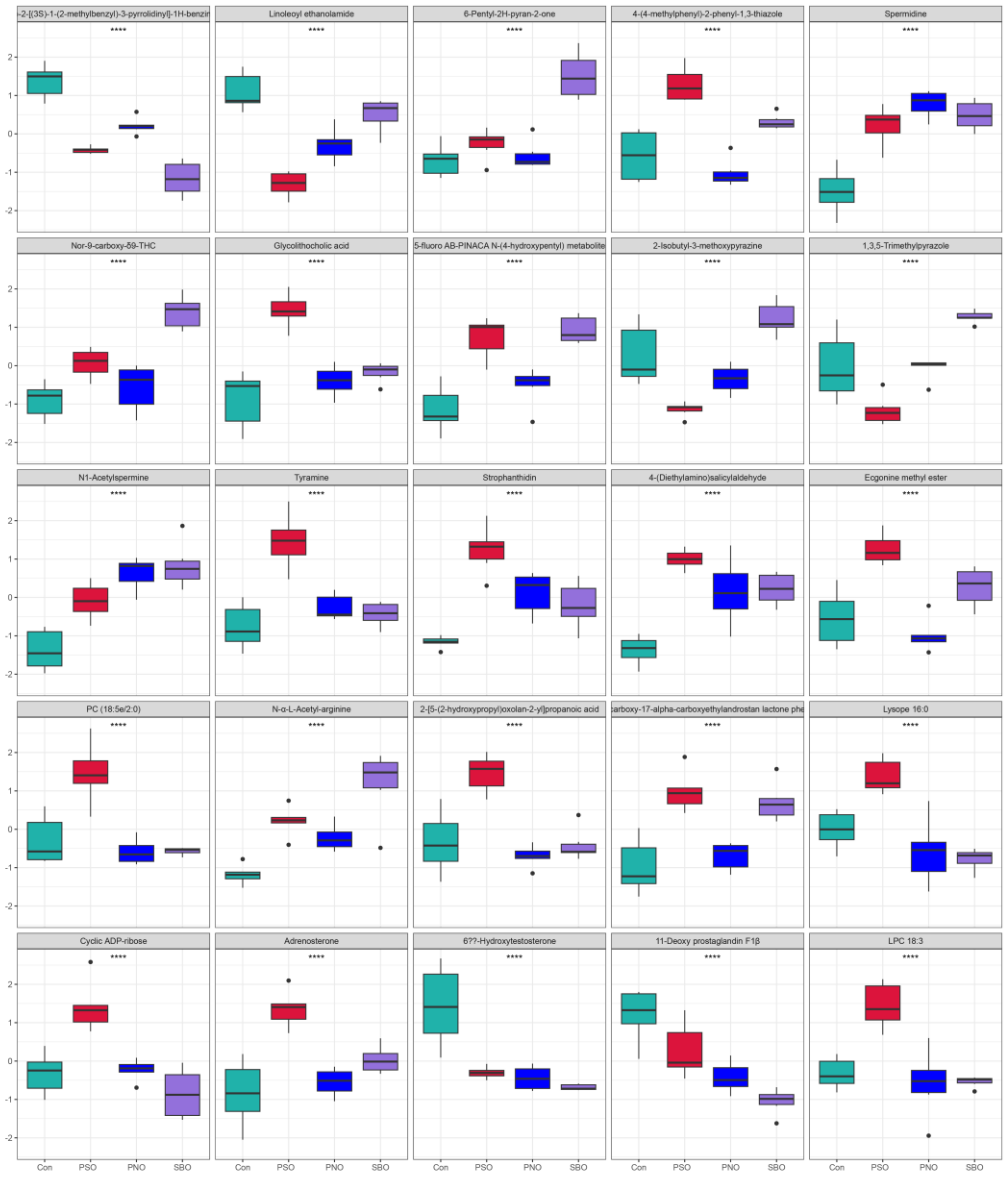

Figure 8 Untargeted metabonomics analysis of SCFAs in positive mode (**** $P < 0.001$).

3.5. Gut metabolome analysis

3.5.1. Metabolic profile analysis

To further assess the effects of metabolites on mice, we performed untargeted metabolomics analysis to detect differences the metabolites in the PSO group, PNO group, SBO group and the Con group. The total ion chromatogram (TIC) of the QC

samples in both positive and negative ion modes is described in Supplementary Figure 1. The principal components analysis (PCA) scores of positive and negative ions showed that the blank group was separated from the PSO group, while the Con group, PNO group and SBO group were cross-aggregated (Figure 9A-B). The OPLS-DA results of positive and negative ions showed that Con group had significant separation from PSO group, PNO group and SBO group (Figure 9C-D). The model demonstrated excellent performance in the positive ion mode, with R^2 Y = 0.97and Q^2 = 0.79. In addition, in the negative ion mode, the model parameters were R^2 = 0.94 and Q^2 Y = 0.70. These values are close to 1, indicating that the model has a high degree of interpretability and good predictive ability. In the permutation displacement tests of OPLS-DA, pR^2Y and pQ^2 are less than 0.05, which also indicates that the model is reliable (Figure 9E-F).

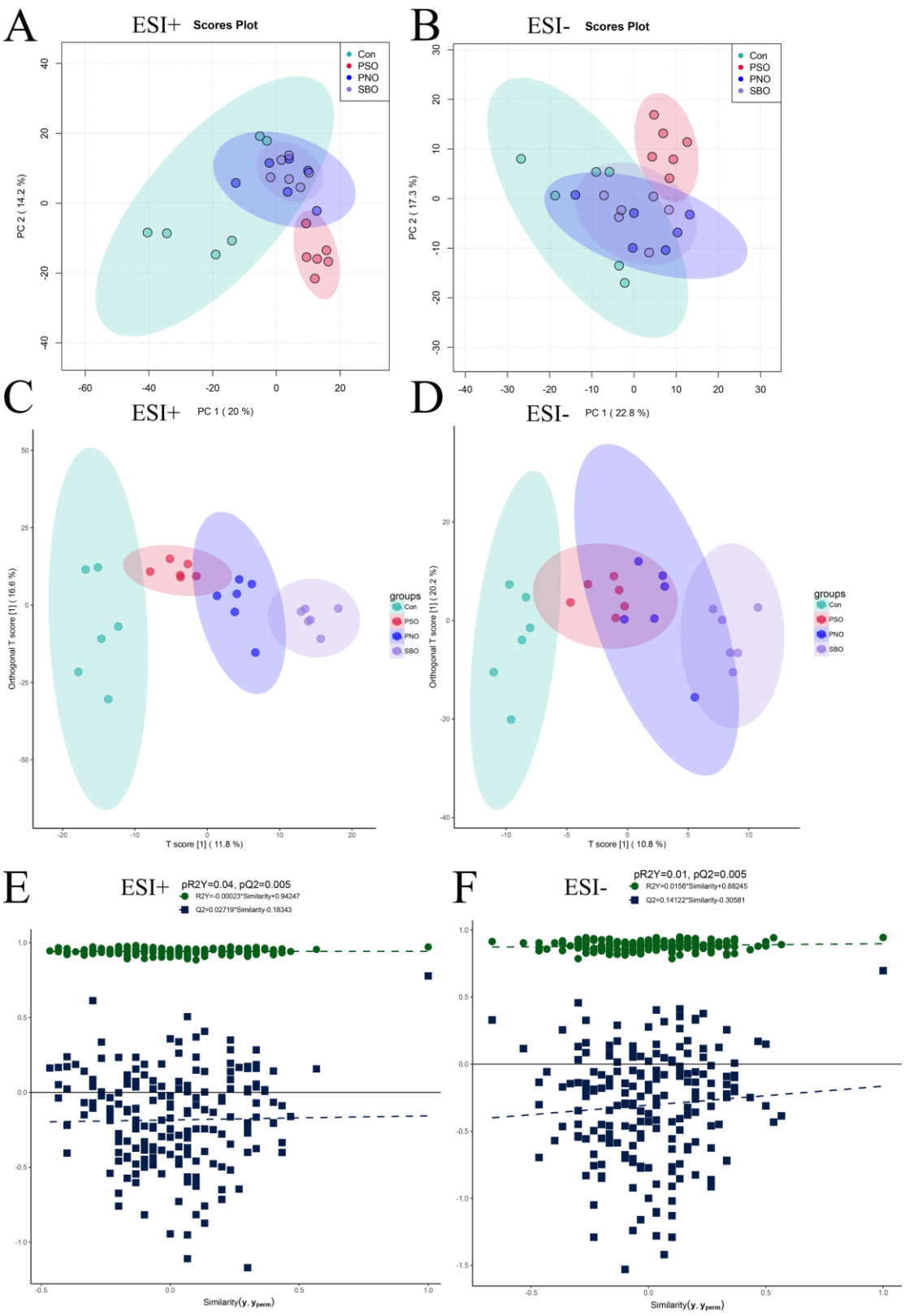

Figure 9 Gut metabolomics analysis of serum in both positive and negative ion modes. ESI + stands for the positive ion mode, and ESI- stands for the negative ion mode. (A-B) PCA analysis score plot. (C-D) OPLS-DA analysis score plot. (E-F) OPLS-DA permutation test analysis plot.

3.5.2. Analysis of the key metabolites and the metabolic pathways

We use hot maps to identify biomarkers with significant differences ($P < 0.05$, $|\log_2 FC| > 2$). We only focus on the top 10 most significant metabolite names (Supplementary Table 2). In positive mode of Con vs PSO, according to HMDB classification, upregulate metabolite included TMK, 3-[(4-hydroxyphenyl)methyl]-octahydropyrrolo[1,2-a]pyrazine-1,4-dione, (Diethylamino) salicylaldehyde, Spermidine, PC (18:4e/2:0), PC (18:5e/2:0). Downregulate metabolite mainly was Linoleoyl ethanolamide, 5-Fluoro-2-[(3S)-1-(2-methylbenzyl)-3-pyrrolidinyl]-1H-benzimidazole, Folinic acid, Pinocembrin, 1,3,5-Trimethylpyrazole (Figure 10A). In negative mode of Con vs PSO, upregulate metabolite comprised tetranor-PGFM, Epicatechin, Catechin, N'-(4-chlorophenyl)-4-ethylbenzohydrazide, 5-Phenylvaleric Acid, Phloretin, D-(-)-Mannitol, cis-Aconitic acid (Figure 10B). In positive mode of Con vs PNO, 4 belonged to others. 2 belonged to lipids and lipid-like molecules. 2 belonged to organic acids and derivatives. 2 belonged to phenylpropanoids and polyketides (Figure 10C). In negative mode of Con vs PNO, 5 belonged to others. 2 belonged to lipids and lipid-like molecules. 1 belonged to organic acids and derivatives. 1 belonged to organoheterocyclic compounds. 2 belonged to nucleosides, nucleotides, and analogues (Figure 10D). In positive mode of Con vs SBO, 2 belonged to organic acids and derivatives. 6 belonged to others. 1 belonged to organic nitrogen compounds. 2 belonged to lipids and lipid-like molecules. 2 belonged to penylpropanoids and polyketides (Figure 10E). In negative mode of Con vs SBO, 3 belonged to others. 1 belonged to benzenoids. 2 belonged to lipids and lipid-like molecules. 1 belonged to organic acids and derivatives. 2 belonged to nucleosides, nucleotides, and analogues. 1 belonged to organoheterocyclic compounds (Figure 10F). The difference of metabolites in different treatment groups was further analyzed by heat map analysis, and the results showed that the composition and structure of these 30 metabolites were significantly different among different groups (Figure 10G-H).

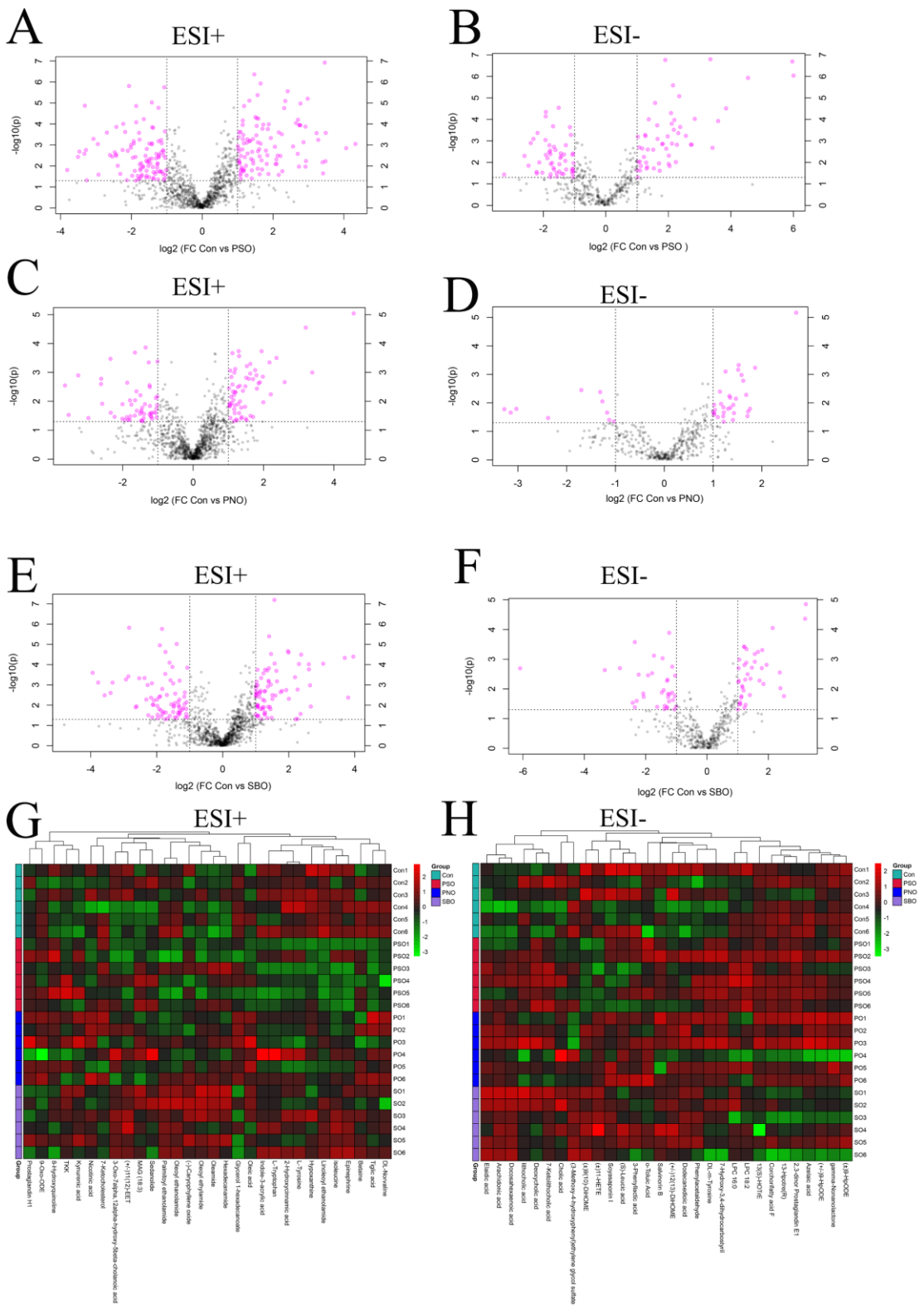

Figure 10 Effects of PSO on intestinal metabolites. (A-B) Volcano plot of Con vs PSO in positive mode and negative mode. (C-D) Volcano plot of Con vs PNO in positive mode and negative mode. (E-F) Volcano plot of Con vs SBO in positive mode and negative mode. (G-H) Heatmap of the top 30 metabolites of 4 groups in positive mode

negative mode.

We perform metabolic analysis by KEGG and calculate the ORA (Over-Representation Analysis) *P*-values of these metabolic pathways to determine whether the metabolites of interest are significantly enriched in these metabolic pathways. In positive mode of Con vs PSO, significant enriched metabolic pathways were nucleotide metabolism, steroid hormone biosynthesis, vitamin B6 metabolism (Figure 11A). In negative mode, Figure 11A showed that the chosen biomarkers are highly involved in the pathway network related to TCA cycle, alanine, aspartate and glutamate metabolism, arginine biosynthesis, glyoxylate and dicarboxylate metabolism and so on. The result indicated that sleep may be connected with these multiple pathways and the potential role of PSO in regulating sleep by influencing key metabolites and the aforementioned metabolic pathways. In positive mode of Con vs PNO (Figure 11C), obvious enriched metabolic pathways were nucleotide metabolism, vitamin B6 metabolism, phenylalanine metabolism, purine metabolism, arginine and proline metabolism. In negative mode, PNO regulated the Biosynthesis of unsaturated fatty acids, linoleic acid metabolism, and nucleotide metabolism pathway (Figure 11D). In positive mode of Con vs SBO (Figure 11E), the remarkable enriched metabolic pathways only were steroid hormone biosynthesis, nucleotide metabolism. And SBO group adjusted 5 pathways in negative mode (Figure 11F).

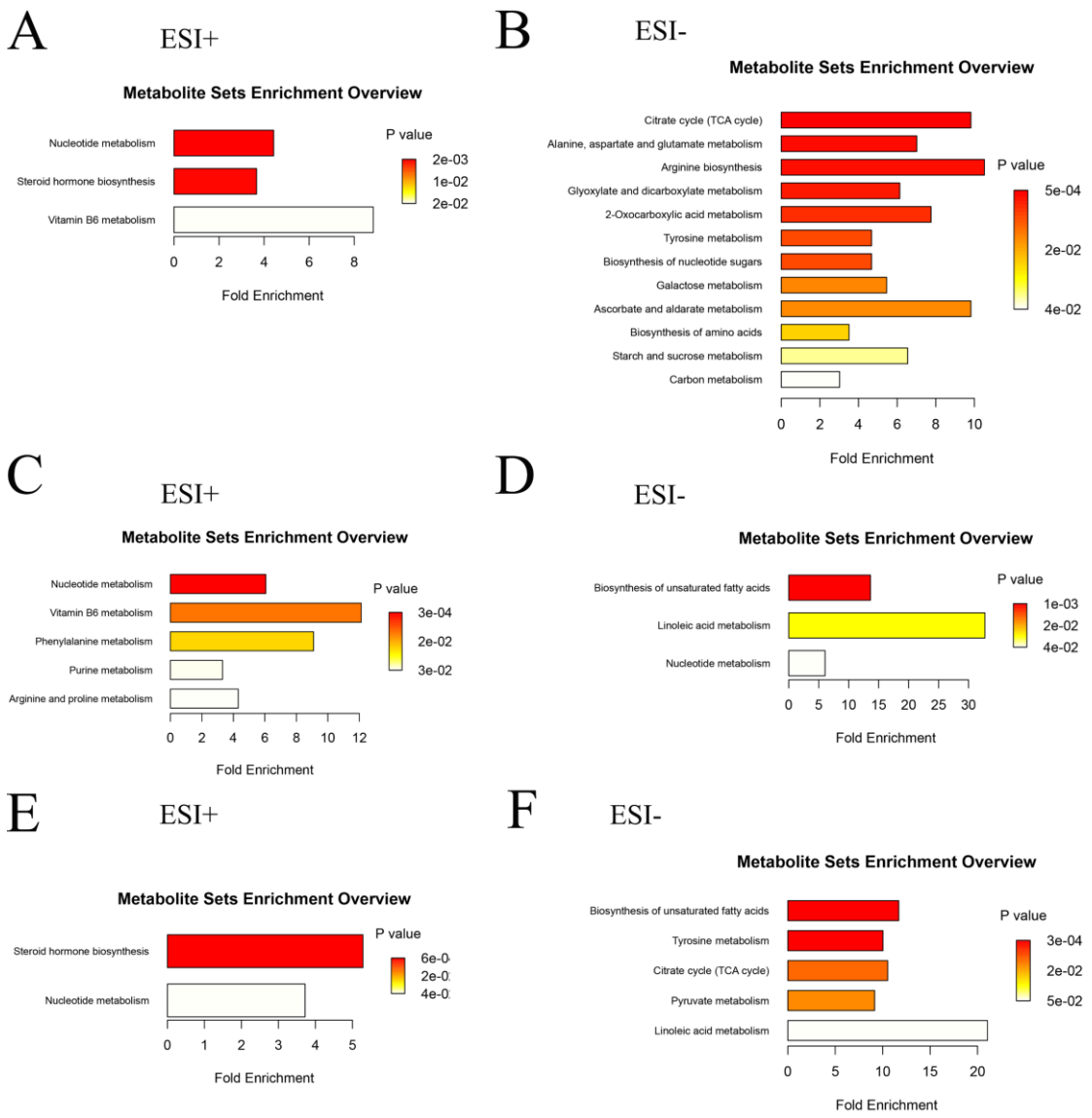

Figure 11 KEGG metabolic pathway enrichment analysis of Con vs PSO in positive mode (A) and negative mode (B). KEGG metabolic enrichment analysis of Con vs PNO in positive mode (C) and negative mode (D). KEGG metabolic enrichment analysis of Con vs SBO in positive mode (E) and negative mode (F). ($P < 0.05$)

3.6. Integration analysis

In order to further explore the relationship among sleep, gut microbes and metabolites, we executed the correlation analysis using Spearman's analysis method. The study performed a correlation matrix linking the top 20 bacteria with the aforementioned 4 sleep indexes. Both sleep time and sleep elongation rate were positively related to Odoribactor, Rikenella and Ruminococcus (Figure 12A, $P < 0.001$). Sleep latency had a positive correlated with Bacterioides, Sutterella and

Parabacteriodes ($P < 0.001$). The association analysis of top 20 bacteria and metabolites found that 11 bacteria were positively correlated with 13 metabolites (Figure 12B, $P < 0.001$). These descriptions manifested that gut microbiota and gut metabolites played an essential role in regulating sleep.

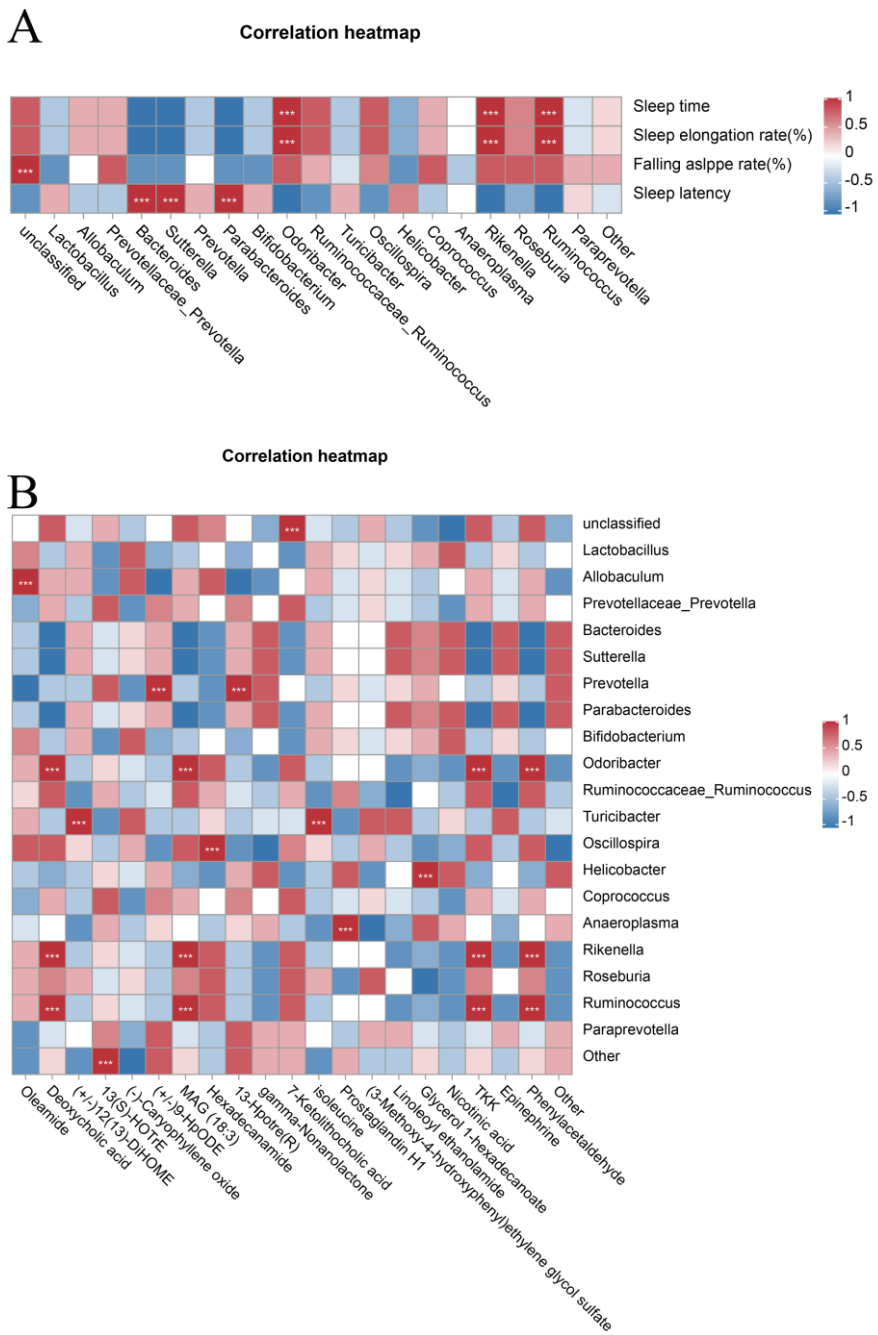

Figure 12 (A) Correlation heatmap between differential genus-level bacteria and sleep indicators. (B) Correlation heatmap between differential genus-level bacteria and differential metabolites (positive and negative ion combined) analysis. ***$P < 0.001$.

4. Discussion

In our study found the PSO had a lot of unsaturated fatty acid, which was up to 90%. Rish unsaturated fatty acid was considered to have the effect of improving sleep quality[14]. In addition, in terms of physiological indexes such as TG, TC, LDL-C, HDL-C and Glu, as well as liver H&E staining indexes, the results of PSO and other edible oils were not significantly different, indicating that PSO had good safety. Many previous articles have focused on alleviating hyperlipidemia and hyperglycemia or anti-obesity on gut microbiome and untargeted metabolomics[15-17]. However, In this study, It is the first time that we analysed the sleep principle of PSO from the perspective of gut microbes and untargeted metabolomics.

In our experiment, PSO group could prolong sleep time and reduce sleep latency. Some studies have shown that 5-hydroxytryptamine (5-HT) and γ-aminobutyric acid (GABA) are involved in sleep regulation[18,19]. Reports indicate that therapeutic drugs cause sedation and hypnosis by modulating neurotransmission pathways, including the 5-HT and GABA systems within the central nervous system[20]. 5-HT regulates sleep by eventually converting into melatonin[21]. But GABA binds to receptors on neurons, it can inhibit the firing frequency of neurons, resulting in sedation and sleep[22]. It has been reported that some components of vegetable oil could promote sleep by elevating 5-HT level and enhance GABA synthesis to promote GABA A receptor to express[23]. Our study adds valuable evidence and reveals new links between PSO and neurotransmitters in sleep.

It has been reported that the gut microbiome is a source of signals that promote sleep[24]. Microbiome diversity and abundance (α and β diversity) are positively correlated with improved sleep quality and increased total sleep duration.[25]. Our experimental results show that the PSO group increased gut richness and diversity in mice. Previous study showed that the abundance of Bacteroidetes and Firmicutes has positive correlationwith sleep quality[26]. A higher proportion of Verrucomicrobia is associated with improved cognitive function[27]. In our experiment, the abundances of Bacteroidetes, Firmicutes, and Verrucomicrobia were relatively high. Lactobacillus spp. regulates sleep disorders and memory by converting the excitatory neurotransmitter

glutamate into the primary inhibitory neurotransmitter GABA through the GABAergic receptor system[28]. Studies have reported that the gut microbiome induces non-rapid eye movement sleep through a butyrate-sensitive mechanism[29]. In PSO group, bacteria such as Oscillibacter, Ruminococcus, Allobaculum, Lachnospiraceae, can participate in butyrate production, which is consistent with previous research findings[30]。

Bacterial metabolites may provide a crucial link between gut symbiotic communities and sleep-generating mechanisms in the brain. Some studies suggest that a higher proportion of fecal SCFA propionate is associated with longer periods of uninterrupted sleep[31,32]. And propionic acid has the ability to influence neurotransmitter concentrations, such as glutamate, GABA, and tryptophan, thereby affecting emotional regulation, cognitive functions, and behavioral patterns[33]. In our experiment, the propionate content in the PSO group was higher than that in other groups. Our untargeted metabolomics results indicate that the PSO group is involved in vitamin B6 metabolism, glutamate metabolism and arginine biosynthesis. Vitamin B6 plays a vital role in the synthesis of several neurotransmitters, including serotonin and GABA, which are critical for promoting sleep[34]. Glutamate is the precursor of GABA and maintains a balance with it[35]. Arginine is converted into nitric oxide in the body, which promotes blood circulation and relaxes blood vessels, potentially aiding in improving sleep quality[36].

In summary, the regulatory mechanism of PSO was investigated in an animal model using metabolomics and gut microbiota analysis. After gavaging with PSO, there were no differences in liver levels and blood markers in mice compared to other edible oils. The mice showed improved sleep quality, increased sleep duration, and decreased sleep latency. PSO significantly altered the gut microbiota of the mice, increasing the abundance of short-chain fatty acids and promoting the production of sleep-promoting metabolites. Further research is necessary to comprehensively understand the precise regulatory mechanisms underlying the influence of PSO.

Data Availability Statement

The data presented in this study are available on request from the corresponding author. Data sets generated and/or analyzed in the current study are available in supplementary information.

Support information

Supporting information may be found in the online version of this article.

Conflicts of interest statement

The authors have no conflicts of interest.

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