Pure total flavonoids from citrus attenuate non-alcoholic fatty liver disease in mice via the gut microbiota

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Research article

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Abstract

Background An increasing number of studies suggest that gut microbiota composition and structure contribute to the pathophysiology of non-alcoholic fatty liver disease(NAFLD),and the gut microbiota has been proposed as a new target in the treatment of diet-induced NAFLD. In this study, we aimed to investigate the effects of pure total flavonoids from citrus (PTFC) on NAFLD and gut microbiota dysbiosis in high-fat diet (HFD)-fed mice and to further investigate whether the attenuation of NAFLD is related to the modulation of the gut microbiota. Results PTFC intervention could significantly attenuate symptoms in mice with HFD-induced NAFLD.Based on the results of 16S rDNA sequencing, PTFC treatment could increase the phylogenetic diversity of the HFD-induced microbiota. PTFC intervention could significantly restore the HFD-induced increases in the relative abundances of Bacteroidaceae and Christensenellaceae. Conclusion Our results suggested that PTFC could serve as a novel candidate for the prevention of NAFLD by modulating the gut microbiota.

Background

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide, affecting up to 25% of adults and children[1–3].The spectrum of NAFLD ranges from simple nonalcoholic fatty liver (NAFL) to non-alcoholic steatohepatitis(NASH), which can progress to liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC)[4, 5].NAFLD is strongly associated with obesity and obesity-related metabolic disorders[6, 7]. NAFLD pathogenesis was initially explained by the two-hits hypothesis, but this theory failed to explain various NAFLD-related molecular changes, resulting in the adoption of the multiple-hits hypothesis,which takes into account the complex and multifactorial aspects of the disease[8, 9].

A large amount of evidence has revealed that the gut microbiota plays vital roles in the regulation of NAFLD by producing bacterial metabolites such as short-chain fatty acids, indole and its derivatives,secondary bile acids, and trimethylamine[10–12].

Pure total flavonoids from citrus (PTFC) were isolated and purified from dried and mature peel of Citrus Varieties (Changshan Huyou) in Changshan and Quzhou of Zhejiang province,China. PTFC is composed of naringin, neohesperidin and narirutin, the total content of flavonoids is over 76%. Naringin can reduce lipid synthesis, improve lipid deposition and reduce inflammatory cell infiltration in the liver of rats induced by high-fat diet [13–15]. The bioactive naringin is the main metabolite of naringin in human intestinal microbial environment, which indicates that the microbiota play an important role in regulating the pharmacological action of naringin[16].Our previous study showed that PTFC controlled the progression of NAFLD by regulating the TLR/CCL and VEGF-C signalling pathways[17–18].

In the present study, we examined whether PTFC could attenuate features of HFD-induced NAFLD in mice, which might be related to the modulation of the gut microbiota.

Results

PTFC ameliorates hepatic steatosis and fibrosis in HFD-fed mice

In our previous study[16], we reported that compared with the ND group, the liver tissues of mice in the HFD group showed obvious steatosis, inflammatory cell infiltration, and focal necrosis. Moreover, fibrous tissue deposition around the perisinusoidal and liver cells also occurred,and the NAFLD activity score (NAS) increased significantly. Compared with the HFD group, the HFD + PTFC group showed a decrease in hepatic steatosis, inflammatory cell infiltration, and degree of fibrosis as well as NAS.

Overall structural modulation of the gut microbiota after PTFC treatment

16S rDNA sequencing analysis was used to investigate the structural changes in the gut microbiota in mice that received PTFC treatment. In total, 644541 useable reads were obtained from 15 samples, and 678 OTUs were collected. Similar to the previous findings, the diet was found to be a key factor in changing phylogenetic diversity. In this work, significant decreases in Chao1 and ACE, indicating lower microbiota community diversity, were found in the HFD and PTFC groups ($P < 0.01$, Fig.1A-C). The rank-abundance curve,rarefaction curve,sample Shannon curves and species accumulation curves show that the amount of sequencing data is reasonable (Fig. 1D-G).

Lefse analysis

Different colours represent different groups. For example, the red node in the branch represents the species with a significantly higher abundance in the red group, the green node represents the species with a significantly higher abundance in the green group, and the yellow node represents the species without a significant abundance difference between groups. The node diameter is directly proportional to the relative abundance; the nodes in each layer represent phyla/classes/orders/families/genera/species, in that order; the annotation of each species represents phyla/class/order/family/genus/species,in that order.The annotation of the phylum level is displayed on the outermost ring, while the annotation information of the other levels is displayed in Fig.2A-B. Bacteroidaceae and Rikenellaceaewere increased in the PTFC group at the family level ($P < 0.05$).Porphyromonadaceae and Enterococcaceaewere increased in the HFD group at the family level ($P < 0.05$).

Venn diagram

From the Venn diagram, we can see the unique OTUs and the common OTUs of each group, indicating the unique species information of each group more directly.Different numbers of OTUs were detected in each group, i.e., 638 in the normal group, 478 in the HFD group, and 458 in the PTFC group (Fig. 2C). Among all OTUs,385 were shared by all groups. Additionally, each group had unique OTUs,including 132 in the normal group,4 in the HFD group, and 21 in the PTFC group.

Analysis of the whole intestinal microbiota composition

At the family level,compared with the normal group, the relative abundance of Christensenellaceae and Erysipelotrichaceaewas decreased in the HFD group. After PTFC treatment, the relative abundance of Christensenellaceae and Erysipelotrichaceae weresignificantly increased(P< 0.01). The relative abundance of Porphyromonadaceae and Streptococcaceae increased in the HFD group. After PTFC treatment, the relative abundance of Porphyromonadaceae and Streptococcaceae were significantly decreased(P< 0.01, P< 0.05)(Fig.3A-D).

At the genus level, compared with the normal group, the relative abundance of Allobacullum was decreased in the HFD group. After PTFC treatment, the relative abundance of Allobacullum was significantly increased(P< 0.05). The relative abundance of Eubacterium was increased in the HFD group. After PTFC treatment, the relative abundance of Eubacterium was significantly increased(P< 0.05)(Fig.3E andF).

At the family level,we found that the majority of the intestinal microbiota consisted of species from Porphyromonadaceae,Lachnospiraceae,Ruminococcaceae,Prevotellaceae,Desulfovibrionaceae, and Bacteroidaceae(Fig.4A). Compared with the normal group, the abundance of Porphyromonadaceae was significantly increased in the HFD group. After treatment with PTFC, the abundance of Porphyromonadaceae in HFD-fed mice decreased(P< 0.01) (Fig.4C-E).

At the genus level, we found that the majority of the intestinal microbiota consisted of species fromClostridium_IV, Bacteroides, Paraprevotella, Helicobacter, Elsenbergiella, and Parabacteroides(Fig.4B). Compared with the normal group, the abundance of Paraprevotella was significantly decreased in the HFD group(P< 0.01). After treatment with PTFC, the abundance of Bacteroides in the PTFC group increased(P< 0.05), andAkkermansiawas identified with anabundance of 4.4%(Fig.4F-H).

PICRUSt Analysis and prediction of genomic functional changes among different groups

Based on the 16S rDNA and reference sequence database, PICRUSt predicted the macrogenome functional composition of the flora. The accuracy of the method was 84-95%. In the PTFC group, 12 KEGG pathways were altered, including energy metabolism, amino acid metabolism, ethylbenzene degradation and peptidoglycan biosynthesis(Fig.4I).Compared with the HFD group, other ion-coupled transporters were upregulatedand beta-alanine metabolism, type I diabetes mellitus, ethylene degradation, energy metabolism, alanine, aspartate and glutamate metabolism were reduced(Fig.4J). These significant pathway changes predicted that PTFC treatment could improve the gut microbiota structure of mice under the condition of HFD feeding, thus exerting a therapeutic effect against NASH in mice.

Discussion

In our previous studies, we found that PTFC treatment could alleviate NAFLD by regulating the TLR/CCL inflammatory signalling pathways, theSIRT1/PGC-1αpathways and Th17/treg balance[15, 17–

20].However, the specific mechanism requires further study. In this study, we aimed to determine the preventive effects of PTFC on high-fat-diet (HFD)-induced NAFLD in mice and the underlying mechanism focusing on gut microbiota profile modulation.

In our study, we found that compared with the normal-diet group, HFD-induced NAFLD micehad differences at the phylum, class, and genus levels that resulted in gut microbiota dysbiosis. These changes were characterized by an increase in the Firmicutes/Bacteroidetes ratio and a dramatic decrease in the Christensenellaceae family compared to that in the normal diet group.

To study the underlying mechanism of PTFC in treating NASH, we investigated the positive effect of PTFC on the intestinal microbiota. In this study, as shown in Fig. 4, at the family level,compared with thenormal group, the relative abundance of Christensenellaceae and Erysipelotrichaceaewas decreased in the HFD group. After PTFC treatment, the relative abundance of Christensenellaceae and Erysipelotrichaceae were significantly increased. The relative abundance of Porphyromonadaceae and Streptococcaceaeincreased in the HFD group. After PTFC treatment, the relative abundance of Porphyromonadaceae and Streptococcaceae was significantly decreased. Christensenellaceae, a recently described family in the phylum Firmicutes, is emerging as an important player in human health[21].Christensenellaceae is reported to bereduced in individuals with metabolic syndrome (MetS) compared to the level in healthy controls [22]. Stachyose improved the intestinal homeostasis of HFD-fed mice by improving the bacterial diversity with increases in the relative abundance of Christensenellaceae[23]. Erysipelotrichaceae was negatively correlated with body weight,while Porphyromonadaceae was positively correlated with body weight[24]. At the family level,Streptococcaceae was enriched in the NAFLD group[25, 26].

At the genus level, after treatment with PTFC, the abundance of the beneficial bacteria with protective effects in the body increased. The abundance of Bacteroides in the PTFC group increased, and Akkermansiaincreased at a ratio of 4.4%.Akkermansia has been inversely associated with obesity, diabetes, inflammation, and metabolic disorders[27, 28]. Due to its highly promising probiotic activities against obesity and diabetes, Akkermansia has drawn intense interest in the context ofresearch and development in recent years[29].

To verify the metabolic function of the intestinal microbiota, the KEGG database was used to identify the relationship between metabolic function and host metabolites (Fig. 4I,J). In the PTFC group, 12 KEGG pathways were altered, including energy metabolism, amino acid metabolism,ethylene degradation and peptidoglycan biosynthesis (Fig. 4I).Compared with the HFD group,other ion-coupled transporters were upregulated, and beta-alanine metabolism, type I diabetes mellitus,ethylene degradation, energy metabolism, and alanine, aspartate and glutamate metabolisms were reduced(Fig. 4J).These significant pathway changes predicted that PTFC treatment could improve the gut microbiota structure of mice under the condition of HFD, thus exerting a therapeutic effect on NAFLD in mice.

Conclusions

This study first analyzed the composition of gut microbiota in NAFLD mice induced by high-fat diet treated with PTFC. The results showed that PTFC increased the richness of the intestinal flora, adjusted the structure of the gut microbial community, and increased the relative abundances of the Bacteroideae,Christensenellaceae and Akkermansia. Therefore, our results indicate that the gut microbiota plays an important role in the progression of NAFLD, and PTFC can regulate this target to play an important therapeutic role.

Methods

Study Animals

All experimental procedures were approved by the Animal Ethics Committee of Zhejiang Chinese Medical University (ZSLL-2016-134)and the National Guidelines for Experimental Animal Welfare. A total of 18 C57BL/6J mice (6-8 weeks of age, male) were purchased from Shanghai Sippr-BK Laboratory Animal Co. Ltd. (production license: SCXK (Hu) 2013-0016). Animals were housed in individually ventilated cages, provided with water and standard/high fat chow, and monitored daily for health and clinical signs. All mice were randomly divided into three groups: a normal diet (normal), a high-fat diet (high) and PTFC. In the 7th week,the PTFC group was fed with 50 mg/kg/d PTFC for an additional ten weeks. At the endpoint of 16 weeks, C57 mice were anesthetized with sodium pentobarbital(50mg/kg),sodium pentobarbital was chosen because it is an approved anesthetic method, fast-acting, reliable, and commonly used in animal studies[30]. The dose of it was chosen to be the minimal recommended dose to achieve the anesthetic effect. The animals were euthanized from exsanguination，blood samples were extracted intraocularly, and fresh stool samples were collected and stored at -80℃ until further analysis.

Preparation of PTFC

Pure total flavonoids from citrus were prepared as previously described [16]. PTFC were separated and purified from *Citrus Changshan-huyou*Y.B.Chang by HPD-300 macroporous resin. The main flavonoids were naringin, neohesperidin and naringin. The total flavonoidcontent was 76.22%.

Gut microbiota analysis

DNA fromfaecalsamples was extracted using aQIAamp® DNA Stool Mini Kit (Qiagen, Hilden,Germany). The resulting DNA was amplified withspecific barcoded bacterial primers targeting the V3-V4 hypervariable region of the 16S rDNA gene using primers:forward primer (5-CCTACGGGNGGCWGCAG-3) and reverse primer (5-GACTACHVGGGTATCTAATCC-3). For each faecal sample, sequencing and bioinformatics were carried out by Genesky Biotechnologies Inc. (Shanghai, China) on the Illumina MiSeq platform to generate 2 × 250 bp paired-end reads. The remaining unique reads were clustered into operational taxonomic units (OTUs) based on the Ribosomal Database Project (RDP) by UPARSE with a 97% similarity cutoff after the raw reads were quality filtered and merged by fastx, Cutadapt, Usearch and FLASH.Mothur was used to perform rarefaction analysis and calculate the community richness index and alpha diversities, including the Shannon and Simpson indexes. Sample tree cluster by Bray-Curtis

distance matrix and an unweighted pair-group method with arithmetic means (UPGMA) and Jaccard principal coordinate analysis (PCoA) based on OTUs were performed using R Project (Vegan package, V3.3.1). Redundancy analysis (RDA) was analysed by Canoco for Windows 4.5 (Microcomputer Power, NY, USA), which was assessed by MCPP with 499 random permutations.

Statistical analysis

Data are presented as the means ± standard errors of the mean. The significant differences between and within the different groups were examined using one-way or two-way ANOVAs, followed by Duncan's multiple-range test. $P < .05$ was considered statistically significant.

Abbreviations

NAFLD: non-alcoholic fatty liver disease;

NAFL: nonalcoholic fatty liver;

NASH: non-alcoholic steatohepatitis;

HCC: hepatocellular carcinoma;

PTFC: Pure total flavonoids from citrus;

PCA: principal component analysis;

PCoA: principal coordinates analysis;

OUT: operational taxonomic unit;

KEGG: Kyoto Encyclopedia of Genes and Genomes;

PICRUSt: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States

Declarations

Consent for publication

Not applicable.

Author's contributions

ZC and SZ designed the study and revised the manuscript. BH and JJ analyzed the data and wrote the manuscript. ZS,DC,SX,MY performed the experiments. All authors read and approved the final manuscript.

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Availability of data and materials

The dataset analyzed during the current study is available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All experimental procedures were approved by the Animal Ethics Committee of Zhejiang Chinese Medical University and the National Guidelines for Experimental Animal Welfare.

Competing Interests

The authors declare that they have no competing interests.

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Figures

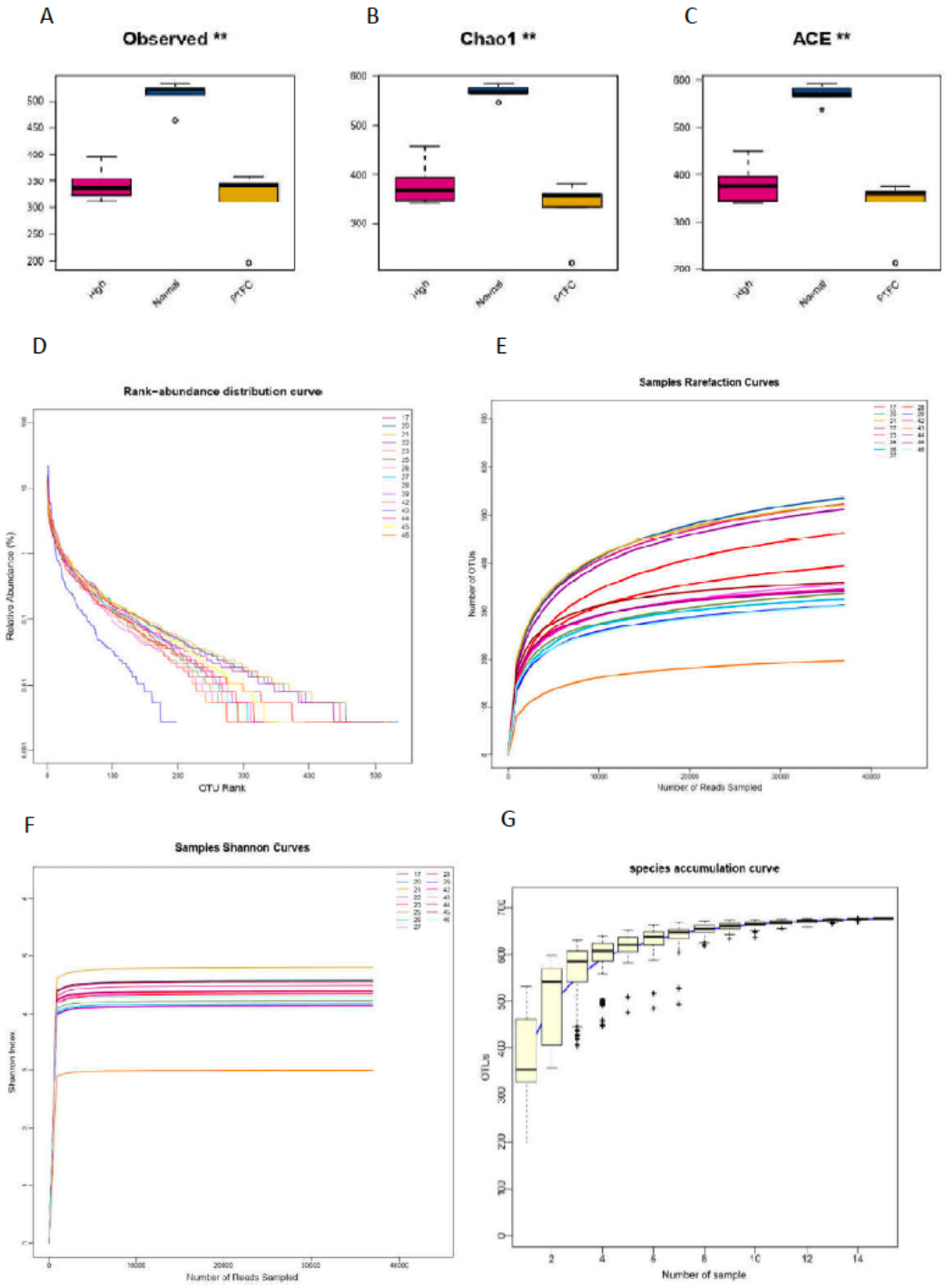

Figure 1

Alpha Diversity. (A)Observed(B)Chao1(C)ACE(D) Rank-abundance distribution curve(E) Samplerarefaction curves(F) Sample Shannon curves(G) Species accumulation curve.

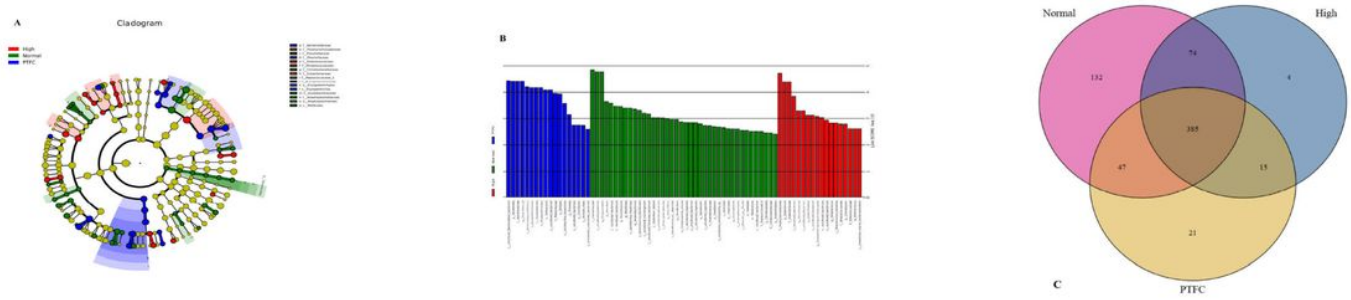

Figure 2

LEfSe Analysis (A)taxon.stat.cladogram (B)taxon.stat.res.Venn diagrams describing the number of operational taxonomic units that are distinct and shared across the groups (C) Venn diagrams.

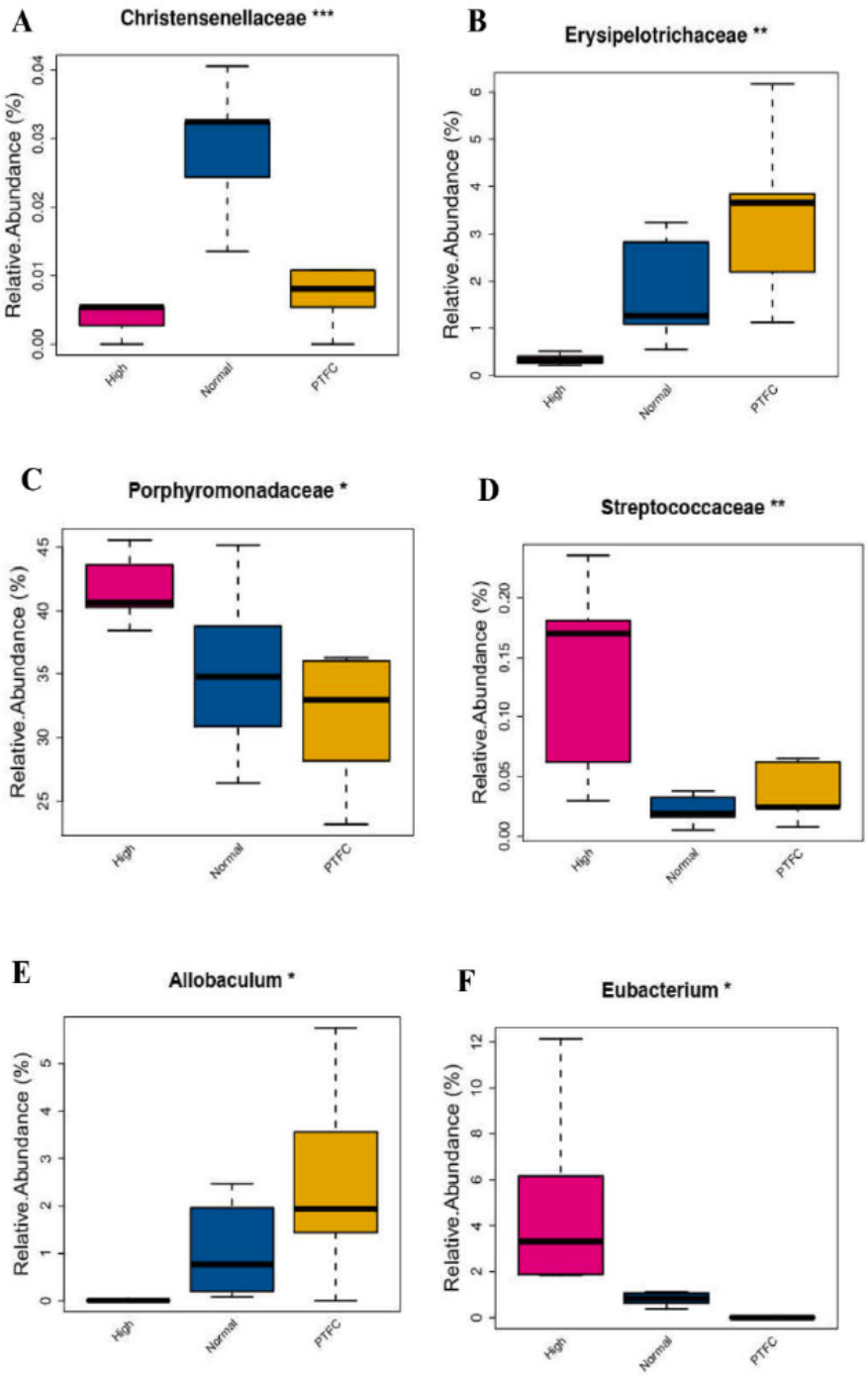

Figure 3

Analysis of the whole intestinal microbiota composition. At the family level, (A) the relative abundance of Christensenellaceae, (B) the relative abundance of Erysipelotrichaceae, (C) the relative abundance of Porphyromonadaceae, and (D) the relative abundance of Streptococcaceae.At the genus level,(E) the relative abundance of Allobacullum (F) and the relative abundance of Eubacterium.

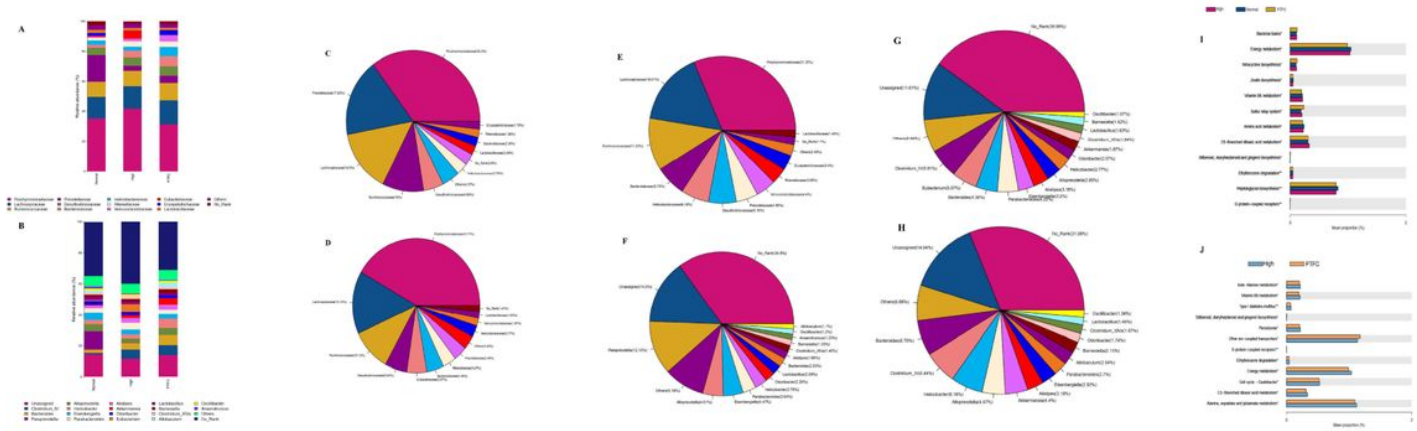

Figure 4

HFD-induced alteration of the gut microbiota in NAFLD mice. (A) Relative abundance of the major bacteria at the family level in the three groups. (B) Relative abundance of the major bacteria at the genus level in the three groups.Community composition analysis in the three groups.(C)Normal.family.pieplot, (D)High.family.pieplot, (E)PTFC.family.pieplot, (F)Normal.genus.pieplot, (G)High.genus.pieplot, and (H)PTFC.genus.pieplot. KEGG (I)KEGG_ANOVA_diff_0.05 and(J)group_different_analysis KEGG.

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