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Regulatory effects of Poria cocos polysaccharides on gut microbiota and metabolites: Evaluation of prebiotic potential

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Abstract

Dietary polysaccharides have the function of regulating gut microbiota and metabolites, and can serve as an important source of functional foods. This study aimed to analyze the regulation of *poria cocos* polysaccharides on the structure and metabolism of human gut microbiota through an *in vitro* fecal fermentation model. The results showed that *Poria cocos* polysaccharides can be utilized by gut microbiota, resulting in a significant decrease in pH and a significant increase in the concentration of short-chain fatty acids. Additionally, the relative abundance of beneficial bacteria such as *Lactobacillus*, *Bifidobacterium* increased, while the relative abundance of harmful bacteria such as *Escherichia-Shigella*, *Bilophila* decreased. Furthermore, *Poria cocos* polysaccharides had a positive effect on microbial metabolites, significantly up-regulating metabolites such as L-cystine, and etelcalcetide. These results highlight the beneficial role of *Poria cocos* polysaccharides in maintaining intestinal health and indicate their potential prebiotics in the functional food industry.

Introduction

Poria cocos is a functional, edible, medicinal fungus widely cultivated and utilized in East Asia [1,2]. It contains a plethora of bioactive compounds, over 198 of which have been identified [3]. Among these compounds, *Poria cocos* polysaccharide (FL) accounts for approximately 70–90% of the dry weight of sclerotia, making it the most abundant substance in the fungus [3]. *Poria cocos* is extensively used as a medicinal and food homology herb in the production of medicinal, food, and health products.

FL are carbohydrates that are composed of ribose, arabinose, xylose, mannose, glucose, and galactose [2]. It possesses a range of pharmacological activities, including anti-inflammatory, antioxidant, antitumor, anti-aging, anti-hyperglycemic properties [4,5]. Polysaccharides are difficult to digest in the small intestine following oral administration and serve as potential prebiotics by microorganisms, thus influencing the composition and metabolism of the gut microbiota [1]. Gut microbiota is the most abundant microbial community in the human body and is an important bridge between dietary nutrient factors and disease. According to research reports, gut microbiota dysbiosis is closely related to the development of a variety of diseases such as obesity [6], constipation [7], and colitis [8]. Thus, the structure of the gut microbiota affects the health of the host, and many diseases may occur that may be related to gut microbiota disorders [9]. Consequently, the gut microbiota and its metabolites can be utilized as targets to alleviate or treat diseases [10].

In recent years, more and more studies have shown that plant polysaccharides, particularly those derived from traditional Chinese medicine, can selectively stimulate the growth and reproduction of beneficial bacteria, such as *Bifidobacterium* and *Lactobacillus*. These polysaccharides activate beneficial bacteria to produce beneficial metabolites, helping to maintain normal physiological functions of the body, and playing a role in immune regulation and disease prevention. Studies have shown that polysaccharides derived from *Gastrodia elata* can stimulate the growth of probiotics, such as *Bifidobacterium*, *Collinsella*,

Prevotella, and *Faecalibacterium*, while inhibiting the abundance of pathogenic bacteria, such as *Shigella*, *Dorea*, *Desulfovibrio*, and *Blautia*. As a result, they act as prebiotics and help to maintain human health [11]. Similarly, polysaccharides extracted from *Ganoderma lucidum* have been shown to alleviate obesity and reduce chronic inflammation and insulin resistance in mice. This is mainly due to the important role played by polysaccharides in regulating the intestinal flora of mice. This suggests that *Ganoderma lucidum* polysaccharides are potential prebiotics for the treatment of obesity and its complications [12]. However, the mechanism of the regulatory effects of FL on gut microbiota and metabolites has not been comprehensively investigated. Further research in this area would be of great significance for promoting host health and preventing diseases.

In this study, 16S rRNA sequencing and non-targeted metabolomics were used to elucidate the regulatory effects of FL on the intestinal flora and its key metabolites. And assess their potential as prebiotics, it aims to provide data support and theoretical basis for the development and application of FL in functional foods.

Results and discussion

Changes in pH and SCFAs

The pH changes observed during *in vitro* fermentation reflect the degree of degradation and utilization of dietary polysaccharides by intestinal microorganisms. The pH changes during the fermentation of FL are shown in Fig. 1A. The initial pH value of the FL was not significantly different from that of the control. There was no significant change in the pH during the fermentation process in the control group. However, as fermentation progressed, the pH value of FL significantly decreased, and after 24 h, the decrease was slow and tended to stabilize without significant changes. During *in vitro* fermentation, the pH of FL was significantly lower than that of the control. The decrease in pH may be related to the utilization of carbohydrates by gut microbes to produce metabolites [13]. Specifically, the accumulation of SCFAs can reduce pH [14]. The total SCFA content increased significantly as the fermentation progressed, as shown in Fig. 1B. This indicated that the gut microorganisms could metabolize FL to produce SCFAs, particularly with the significant enrichment of acetic acid and propionic acids (Figure C and Figure D).

SCFAs are the primary byproducts of colon fermentation of indigestible carbohydrates [15]. Acetic acid is an important source of energy for the brain, heart, and peripheral tissues and is a fermentation product of *Bifidobacterium* and *Lactobacillus* [16,17]. The significant increase in acetic acid content in FL may be related to the increased relative abundance of *Bifidobacterium* and *Lactobacillus* in the FL group (Fig. 4I). Acetic acid is the most abundant SCFA in peripheral circulation and can reduce appetite by crossing the blood-brain barrier through central homeostasis mechanisms [16]. Additionally, it is associated with changes in the expression profile of regulatory neuropeptides, which is beneficial for inhibiting appetite and activating acetyl-CoA carboxylase [18]. Propionic acid can reduce food intake, ameliorate fatty acids [19], and improve insulin sensitivity [20].

In summary, the *in vitro* fecal fermentation of FL stimulates the production of beneficial compounds, reducing the pH of the surrounding environment. These SCFAs have beneficial effects on the colon, gut microbiota, and other organs. Therefore, it is speculated that FL have potential health-promoting effects and could serve as probiotics.

FT-IR analysis

FT-IR was primarily employed to analyze the conformation of glycosidic bonds and functional groups of the polysaccharides. Figure 2 shows the structural characterization of FL fermentation for 0, 6, 12, 24, and 48 h. The absorption peaks appearing near 2,928 cm^{-1} are characteristic groups of polysaccharides, which may be caused by the C-H stretching vibration of the alkyl group [21]. Peak around 1637 cm^{-1} corresponds to the bending vibration of C = O group [22]. The absorption peak in the range of 1000–1200 cm^{-1} is attributed to the tensile vibration of the pyran ring [23]. The characteristic absorption peaks observed near 930 cm^{-1} and 851 cm^{-1} indicate that FL comprise both α and β Glycosidic bonds [24,25]. Moreover, absorption peaks appeared at 774 cm^{-1} at 12, 24, and 48 h, which are characteristic absorption peaks of glucose [26], suggesting that FL contain glucose or have been degraded into glucose by intestinal microorganisms during fermentation. In summary, the FT-IR spectra at different time points in the fermentation process are similar, indicating that the intestinal microorganisms only degrade and utilize FL during fermentation and have no significant impact on its structural characteristics.

Diversity analysis of gut microbiota

Polysaccharides are valuable dietary components with the ability to regulate intestinal flora and improve host health [16,27]. To assess whether the sequencing amount was sufficient to reflect the diversity of species in a sample, sparse curves were used as an indirect measure of species abundance in a sample [28]. The sparse curves of ASV levels Sobs and Shannon indices showed that the amount of sequencing information was sufficient to analyze gut microbial diversity (Figs. 3A and 3B). The Coverage index reflects the coverage of the community, Chao and Sobs indices indicate the richness of the community, and Shannon and Simpson indices represent the diversity of the community [29]. The coverage index of FL and Control was not significantly different and close to 1, indicating that the sequencing results can represent the real situation of microorganisms in the samples (Fig. 3C). As shown in Figs. 3D-3G, the diversity indices Shannon and Simpson are significantly different, while the richness indices Chao and Sobs are not significantly different, indicating that FL affected the diversity of the intestinal flora but not its richness. This is consistent with previously reported results that pectic polysaccharides from okra [30] and citrus pectin [31] have been shown to significantly reduce the diversity of intestinal flora. Similarity assessment was performed based on the bray_curtis algorithm at the ASV level, as shown in Fig. 3H, all samples were clustered into two groups, FL and control. Additionally, Principal Component Analysis was employed to evaluate the differences in the structure and composition of intestinal microflora between the two groups. As shown in Fig. 3I, the PC1 axis and the PC2 axis accounted for 33.59% and 23.71% of

the variance, respectively, and were statistically distinct. In summary, FL have a significant effect on the structural composition of the gut microbiota.

Analysis of gut microbiota composition

Gut microbiota are involved in a variety of roles, such as energy metabolism, nutrient absorption, and immune regulation in the body [27]. Elucidating the relationship between FL and gut microbiota can help prevent disease and promote health by regulating the composition of gut microbiota. At the phylum level (Figs. 4A-4F), primarily consisting of Firmicutes, Proteobacteria, Desulfobacterota, Bacteroidota, Actinobacteriota, and a small amount of Synergistota. The abundance of Firmicutes *was* significantly higher in the FL than in the control, whereas the abundance of Proteobacteria and Desulfobacterota was significantly lower than that in the control. Similarly, *Symphytum officinale* L. polysaccharide was fermented *in vitro*, and the relative abundance of Firmicutes increased [32]. Firmicutes play a vital role in the fermentation of indigestible carbohydrates by producing SCFAs that provide energy to the host and regulate metabolic processes, thus promoting host health [33]. Proteobacteria, the primary phylum of Gram-negative bacteria, contain many pathogenic microorganisms that can disrupt gut microbiota, cause low-grade inflammation, and even colon cancer [16,34].

At the genus level, stacked histograms and heatmap plots were used to represent the relative abundance and composition of species between the two groups (Fig. 4G-4H). It can be visualized from the graphs that the composition of the gut microbiota in the FL was significantly different from that in the control control group. Fusobacterium, a pathogen that proliferates under nutrient-deficient conditions, was inhibited by FL. Compared with the control, the relative abundance of beneficial bacteria, such as *Megamonas*, *Lactobacillus*, *Enterococcus*, *Bifidobacterium*, and *Prevotella*, was significantly increased in the FL group, while the relative abundance of harmful bacteria, such as *Bilophila*, *Lachnoclostridium*, *Escherichia-Shigella*, *Phascolarctobacterium*, and *Klebsiella*, decreased (Fig. 4I). Similarly, Wu et al. found that the fermentation of *snow chrysanthemum* polysaccharides significantly reduced the relative abundance of *Fusobacterium*, *Klebsiella*, and *Escherichia-Shigella* [35]. *Megamonas*, which belongs to the phylum Firmicutes, and can ferment carbohydrates to produce acetic acid and propionic acid, which coincides with the SCFA results (Fig. 1), with correlations to inflammatory bowel disease and nervous system diseases [36]. *Bifidobacteria* and *Lactobacillus* are common microorganisms in the gastrointestinal tract of humans and animals, and play an essential role in maintaining host health. *Lactobacillus* contributes to animal intestinal health by producing various SCFAs [33]. The study found that *in vitro* fermentation of tamarind seed polysaccharides has been found to increase the concentration of SCFAs and the relative abundance of *Lactobacillus* [37]. *Bifidobacteria* is a group of probiotics in the phylum Actinobacteria, are highly beneficial to human intestinal health [38]. To determine the statistically significant characteristic taxonomic groups of FL and the control group, LEfSe combined with linear discriminant analysis (LDA = 4) was used (Fig. 4J-4K). There were 46 characteristic microorganisms at each taxonomic level, ranging from phyla to genera, between the two groups. Among them, 16 microbial

groups exhibiting significant differences were enriched in the FL group, including *Actinobacteria*, *Bifidobacteriales*, *Lactobacillales*, *Megamonas*, and *Prevotellaceae*.

The results indicated that FL can stimulate the proliferation and metabolism of beneficial bacteria in the intestinal tract, inhibit the growth and reproduction of potentially pathogenic bacteria and other harmful bacteria, and at the same time produce beneficial metabolites, such as SCFAs, which was consistent with the concept of prebiotics.

Comparative analysis of metabolites of gut microbiota

Microbial metabolites are important results of dietary nutritional factors regulating intestinal flora. Microbial metabolites can prevent and improve metabolic diseases and maintain human health. We analyzed the metabolites in the FL fermentation broth. The PCA results (Figure. 5A) demonstrated that the QC samples were well aggregated, with a close distance within the group and significant distances between the groups, with an overall explanation degree of 84.70%. The PLS-DA analysis results further showed that the metabolites of the pFL and control were completely separated (Fig. 5B), with significant variability between the control and experimental groups. The PLS-DA model validation results indicated that the intercept of the Q2 regression line with the Y longitudinal axis was − 0.5571, indicating that the model was well fitted and predictable, making it suitable for subsequent data analysis (Fig. 5C).

A comparative analysis of the metabolites of intestinal flora was conducted under positive ionization and negative ion mode using the fermentation broth of FL and a blank group. The results indicated the presence of 3030 and 2965 metabolites in the two groups (n = 3), respectively. Among them, the two groups shared 2733 metabolites. FL contained 2987 unique metabolites, whereas the blank group contained 232 unique metabolites (Fig. 5D). Subsequently, the differential metabolites between the two groups were compared using the HMDB 4.0 database, and these differential metabolites were categorized into 19 categories (Fig. 5E). Organic acids and their derivatives were the most prevalent (27.70%), followed by lipids and lipid-like molecules (23.88%). Other categories included organic chemical compounds (14.22%) and organic oxygen compounds (9.76%). Metabolite cluster analysis demonstrated a clear distinction between the metabolite compositions of different groups (Fig. 5F). The metabolite composition between the two groups exhibited obvious clustering, revealing shifts in the metabolite spectrum of the intestinal flora altered by FL.

Analysis of differential metabolites

Volcano maps directly displayed up-regulated or down-regulated metabolites, and revealed 90 different metabolites between the two groups, of which 50 were up-regulated and 40 were down-regulated (Fig. 6A). The identification of metabolite contributions was facilitated according to the weighting coefficient of the OPLS-DA model, which ranked their contributions based on the VIP score. The top 20 up-regulated metabolites and their contribution order are shown in Fig. 6B, mainly includes L-3-cyanoalanine, tetragastrin, etelcalcetide, adenosine 5’-monophosphate and L-cystine. L-cystine, an amino acid

derivative, possesses antioxidant properties that protect against oxidative stress and tissue damage [39]. Adenosine 5’-monophosphate, a potential anti-obesity agent, has been found to improve obesity [40]. Supplementation with adenosine 5’-monophosphate in the diet has been shown to modulate lipid metabolism in fattening pigs, enhance meat quality, and promote the formation of flavor substances (glutamine, arginine, glycine, and alanine) in muscle tissue [41]. Differential metabolite analysis was performed to examine the effects of FL on the serum metabolic profiles. The top 20 downregulated metabolites were identified (Fig. 6C), including Phe Leu, P-octopamine, xanthine, and pantothenic acid. Xanthine, as a typical purine, can be directly metabolized into uric acid by xanthine oxidase. Elevated levels of uric acid can lead to the formation of sodium urate crystals, which can deposit in joints and cause inflammation, ultimately resulting in gout development [42]. In addition, FL affected 25 KEGG secondary pathways (Fig. 6D), including six main categories: Metabolism, Human Diseases, and Cellular Processes, etc. Among them, metabolism has the most annotated secondary pathways. It mainly includes amino acid metabolism, carbohydrate metabolism, lipid metabolism, and so on. The screened differential metabolites were further analyzed for pathway enrichment (Fig. 6E), with the highest enrichment rate observed in pancreatic cancer, followed by cAMP signaling pathways and choline metabolism in cancer. Additionally, other significant enrichments were observed in renin secretion, antifolate resistance, fat digestion and absorption, cushing syndrome, the phospholipase D signaling pathway, and other metabolic pathways.

Based on these findings, we infer that the biological activity of FL may be partially attributed to their fermentation by the gut microbiota, which increases beneficial metabolites and reduces harmful metabolites. This indicates that they have potential prebiotic activity in the intestine.

Correlation analysis

Microbial metabolites are an important bridge between microorganisms and host health. The relationship between intestinal microorganisms and their metabolites is conducive to understanding the impact of dietary polysaccharides on intestinal flora and host health. This study analyzed the correlation between flora and metabolites, as shown in Fig. 7. The beneficial bacterium *Lactobacillus* displayed a significant positive correlation with metabolites such as raffinose, chondroitin 6-sulfate, panose, core oligosaccharide, maltotriose, L-cystine, etelcalcetide. *Bifidobacterium* was positively correlated with etelcalcetide, adenosine 5’-monophosphate, tetragastrin, L-cystine, and chondroitin 6-sulfate. Raffinose is a trisaccharide composed of D-fructose, D-galactose, and D-glucose, stimulates the growth of *Lactobacillus* and *Bifidobacteria*, and has the potential to be used as a prebiotic [43]. Etelcalcetide is a new calcium mimetic used to treat secondary hyperparathyroidism by directly binding to calcium-sensitive receptors and reducing circulating levels of parathyroid hormone and calcium [44].

Research indicates that panose and chondroitin 6-sulfate exhibited negative correlations with harmful bacteria, such as *Escherichia-Shigella*, *Bilophila*, *Peptostreptococcus*, and *Klebsiella*, while displaying positive correlations with beneficial bacteria, such as *Megamonas*, *Lactobacillus*, *Parabacteroides*, and

Faecalibacterium. Panose has antioxidant and antidiscoloration effects and is a functional sugar with a variety of biological activities [45]. Additionally, panose is also a prebiotic with the ability to regulate the balance of gut microbiota [46]. The metabolite Xanthine demonstrated significant positive correlations with harmful bacteria such as *Bilophila*, *Lachnoclostridium*, *Escherichia-Shigella*, *Dialister*, *Klebsiella*, and *Peptostreptococcus*, while displaying negative correlation with beneficial bacteria such as *Megamonas*, *Lactobacillus*, *Parabacteroides*, and *Faecalibacterium*. Xanthine is generated through the deamination of guanine-by-guanine deaminase and the oxidation of hypoxanthine by xanthine oxidase. Subsequently, it is oxidized by xanthine oxidase to form uric acid [47]. The accumulation of uric acid can result in hyperuricemia, a condition that has become the second most prevalent metabolic disease after diabetes mellitus [48], seriously affecting human health and quality of life. In summary, FL are closely related to intestinal flora and metabolites. FL stimulates gut microflora to produce metabolites by regulating gut microflora, thus affecting the health of the host.

Conclusion

Poria cocos polysaccharide is a type of polysaccharide that exhibits both medicinal and food homology properties and can change the composition of intestinal microflora and metabolites during the fermentation of human feces. At the phylum level, FL have been shown to increase the relative abundance of Firmicutes, while decreasing the relative abundance of Proteobacteria and Desulfobacterota. At the genus level, FL have been found to promote the proliferation of beneficial bacteria and inhibit the growth of harmful bacteria. At the same time, it promotes the enrichment of SCFAs, such as acetic acid and propionic acid, to lower environmental pH and maintain intestinal health. Furthermore, FL significantly enriched beneficial metabolites, such as L-cystine, etelcalcetide, and adenosine 5’-monophosphate, while reducing harmful metabolites, such as xanthine. Further correlation analysis showed that the intestinal flora was closely related to metabolites.

Overall, these findings suggest that FL have the potential to remodel the structure of the intestinal microbiota and regulate metabolites, potentially improving host health. This suggests that FL have prebiotic properties that are beneficial to human health and have potential applications in functional foods.

Materials and methods

Materials and reagents

Poria cocos polysaccharides (90% purity) were obtained from Xi’an Kepin Biotechnology Co., Ltd. The short-chain fatty acids (SCFAs) standards were procured from Macklin Biochemical Technology Co., Ltd. (Shanghai, China).

In vitro fermentation

The preparation of the culture medium is based on previous research reports [49,50], with a pH adjusted to 7.0 ± 0.2, and sterilized at 121°C for 15 min. Fresh feces were collected from four healthy volunteers (n = 4, male: female = 1:1, aged 20–30). The volunteers maintained a plain diet, were in good health, and had no unhealthy habits. Participants signed informed consent forms and completed the survey questionnaires. The study was supervised and approved by Institutional Review Board of College of Food Science, Southwest University. The fecal inoculum was prepared according to a previously described method [50,51].

In vitro fermentation of FL was carried out according to previously published reports [51,52]. Briefly, 1% (w/v) FL was dissolved in the *in vitro* fermentation medium, and the *in vitro* fermentation medium without added FL was used as a control group. The medium was pre-warmed in an incubator at 37°C and inoculated with a 10% (v/v) human fecal homogenate mixture, mixed, and then incubated at 37°C in an anaerobic workstation with three replicates per group. Samples were collected at different time points and pH was determined. All samples were stored at -80°C for further analysis.

Fourier transform infrared spectroscopy (FT-IR)

FT-IR was used to examine the infrared spectra of the samples during fermentation. The supernatant of the fermentation broth was subjected to freeze-drying, and 2 mg samples were weighed for KBr tableting, with a scanning range of 4000 – 400 cm^{-1} [53].

Determination of SCFAs

Fatty acids were extracted using anhydrous ether following a previously described method [54]. The determination of SCFAs was based on the previously described method [55] with slight modifications. An Agilent 8890-5977B gas chromatograph was used for analysis.

Analysis of gut microbiota

The 48 h fermentation precipitate was collected, and total DNA was extracted using the YH-feces stool DNA extraction kit (Shanghai Meiji Suhua Biomedical Technology Co., Ltd.). The V3-V4 region of 16S rRNA was amplified using primers 338F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT) [56]. Sequenced using the Illumina PE300 platform. All data analyses were performed using the Majorbio BioCloud platform (https://cloud.majorbio.com).

Gut microbiota metabolites analysis

The fermentation supernatant (200 μL) was precisely pipetted, and 200 μL of the extraction solution was added to extract the metabolites. The sample injection volume was 3 μL, the flow rate was 0.40 mL/min, the column temperature was maintained at 40°C. Following completion of the computer operation, the LC-MS raw data were imported into the metabolomics processing software Progenesis QI (Waters Corporation, Milford, USA) for handing. Simultaneously, MS and MS/MS information was cross-referenced with the metabolic public databases HMDB (http://www.hmdb.ca/), Metlin (https://metlin.scripps.edu/), and Majorbio self-built databases, and metabolite information was

obtained. All data analyses were performed on the Majorbio Biomedical Cloud Platform (https://cloud.majorbio.com).

Statistical analysis

GraphPad Prism 9.0.2 was used for drawing and representation. The mean ± SD was calculated for all experiments, with three replicates, statistical significance was set at $P < 0.05$. Statistical analysis was performed using SPSS 20.0. The correlation between microorganisms and differential metabolites was analyzed using the Spearman's rank correlation test [57]. Additionally, the Chiplot(https://www.chiplot.online/)tool was used to draw a correlation heatmap.

Declarations

Data availability

The data from the current study are available in the main text.

Author Contribution

X. Zh and Y.Y. L. Designed, Methodology, Software, Writing-original draft, Writing-review & editing. Y.H. Y., L.W., J.H. X. Methodology. Ch. W. Conceptualization, Writing-original draft. J.J. S. Conceptualization, Investigation. Sh.J. L. Investigation. J. Y. B. Conceptualization, Supervision, Validation. H.Y. S. Funding acquisition, Supervision, Writing-original draft, Writing-review & editing. All authors reviewed the manuscript.

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Figures

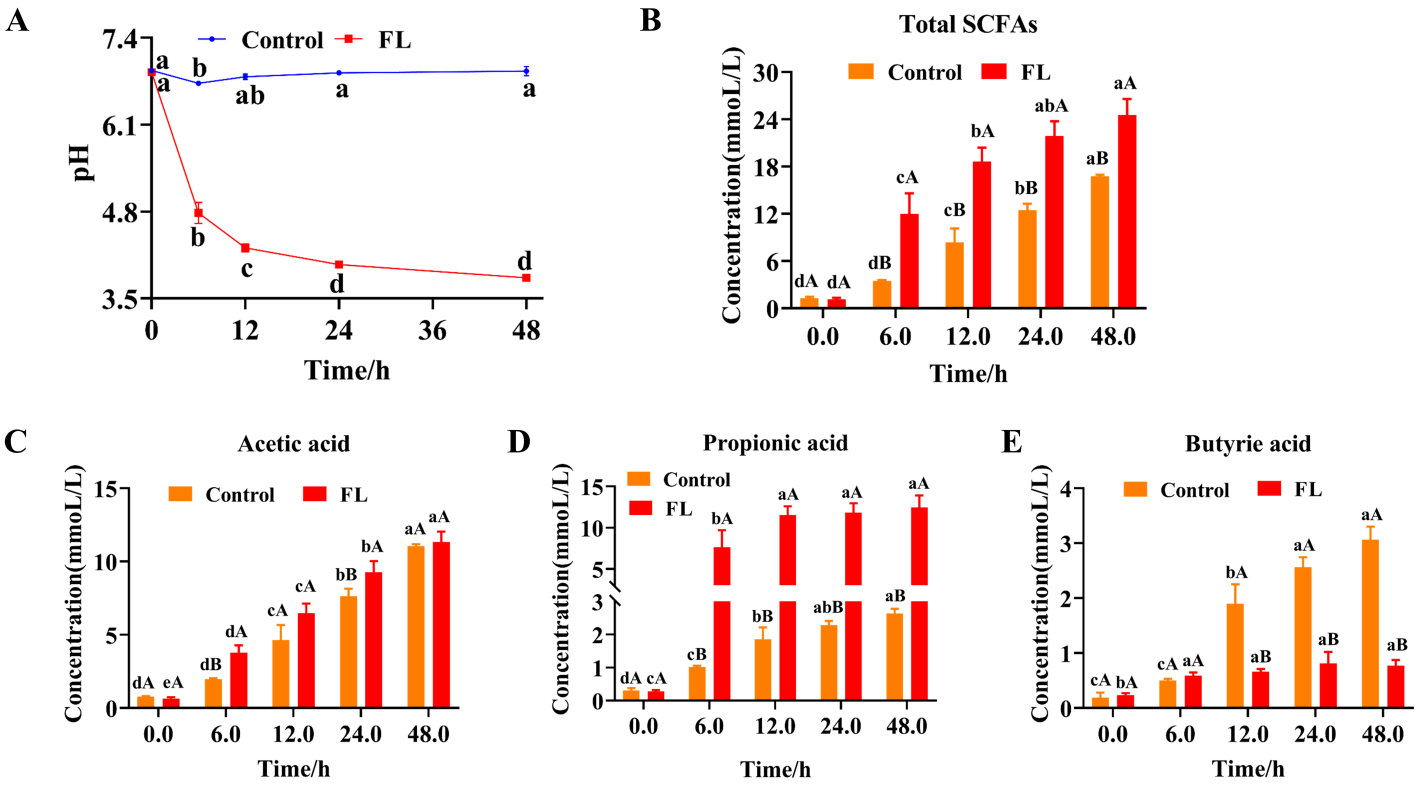


Figure 1

Changes in pH and SCFAs during fermentation. (A) Changes in pH. (B) Changes in Total SCFAs. (C) Changes in acetic acid. (D) Changes in propionic acid. (E) Changes in the butyric acid.

Note: Total SCFAs are the sum of acetic, propionic, and butyric acids. Different lowercase letters indicate that the same sample has significant differences at different times (P<0.05), and different uppercase letters indicate significant differences between the two groups at the same time (P<0.05).

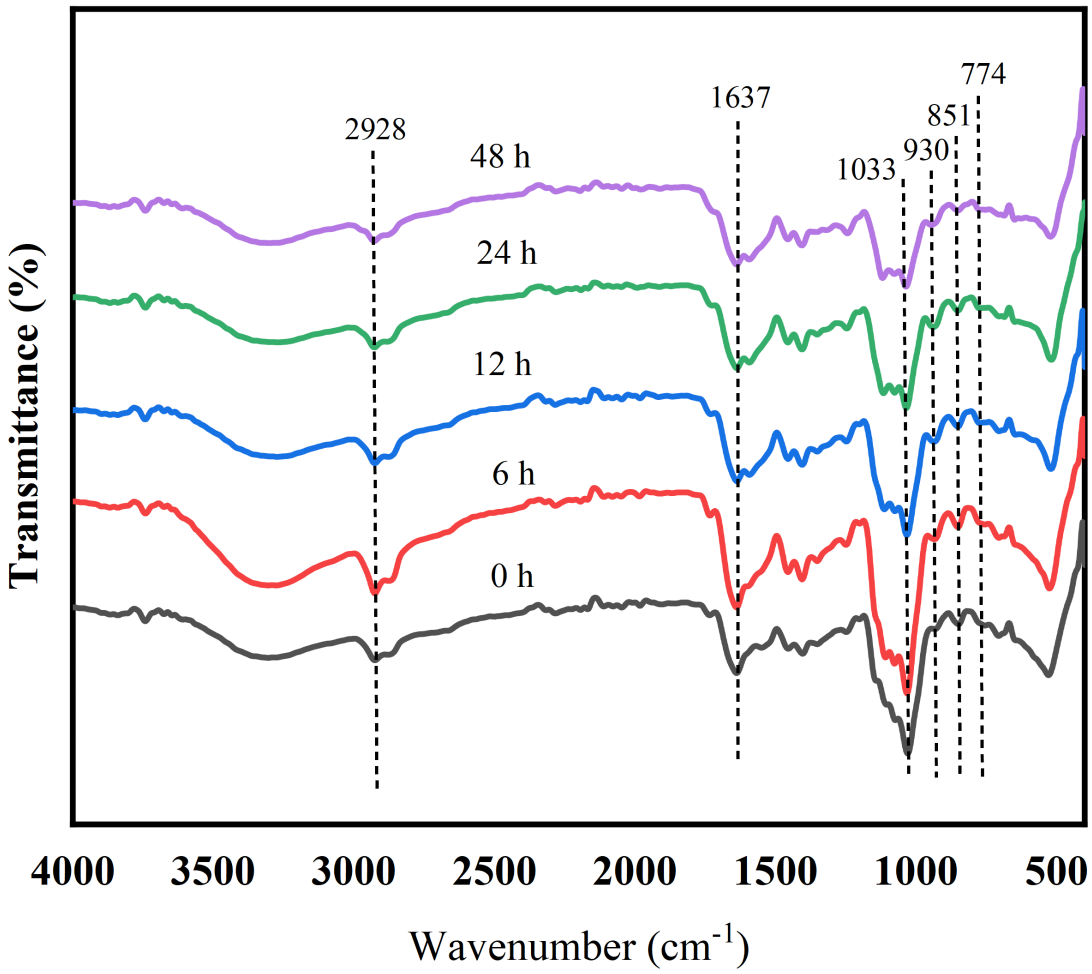


Figure 2

Dynamic changes in FT-IR spectra during *in vitro* fermentation

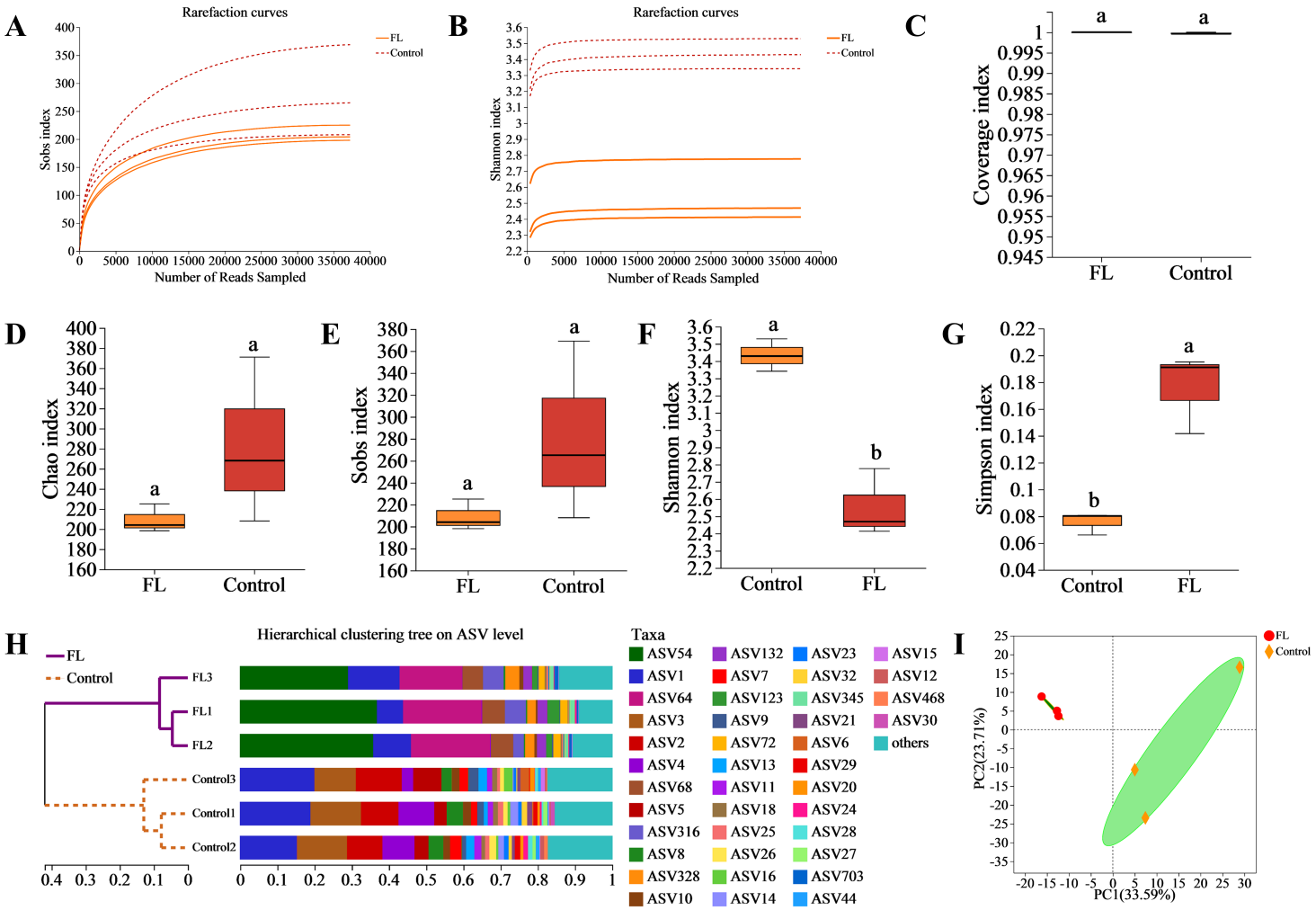


Figure 3

Diversity analysis of intestinal flora. A. Sobs rarefaction curve at the ASVs level. B. Shannon rarefaction curve at the ASVs level. C. Coverage index of ASV level. D. Chao index of ASV level. E. Sobs index of ASV level. F. Shannon index of ASV level. G. Simpson index of ASV level. H. Hierarchical clustering of ASV levels. I. PCA based on the ASV level.

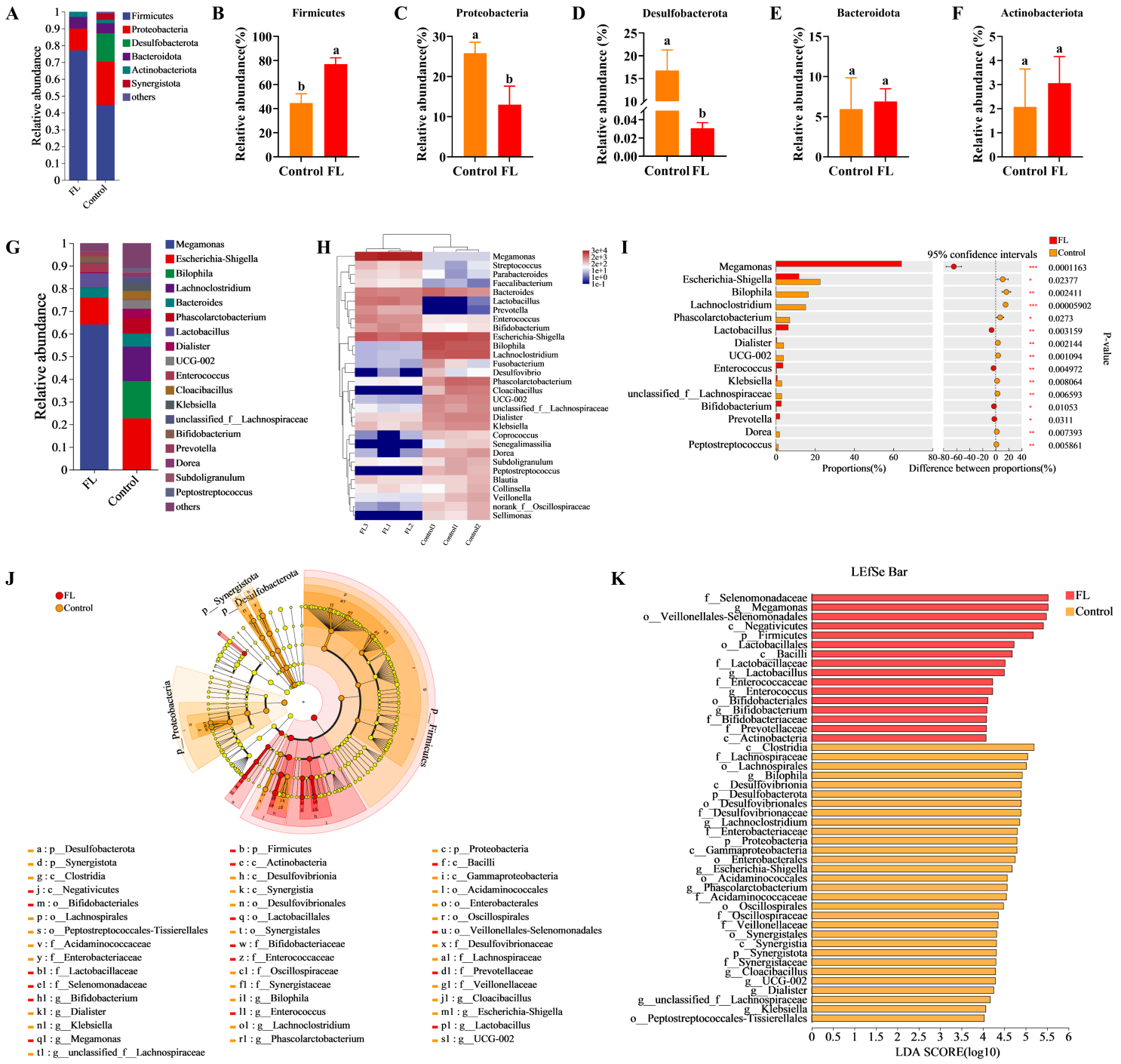


Figure 4

Analysis of gut microbiota composition. A. Community composition between the two groups at the phylum level. B. Comparison of relative abundance of Firmicute. C. Comparison of the relative abundances of Proteobacteria. D. Comparison of relative abundance of Desulfobacterota. E. Comparison of relative abundance of Bacteroidota. F. Comparison of relative abundance of Actinobacteriota. G. Relative abundance between two groups at the genus level. H. Heatmap of relative abundance between the two groups at the genus level. I. Significant differences in Microbial groups

between the two groups. J. LEfSe compared the gut microbiota between the control group and the FL group. K. LDA score for taxa differing between the control and FLgroups.

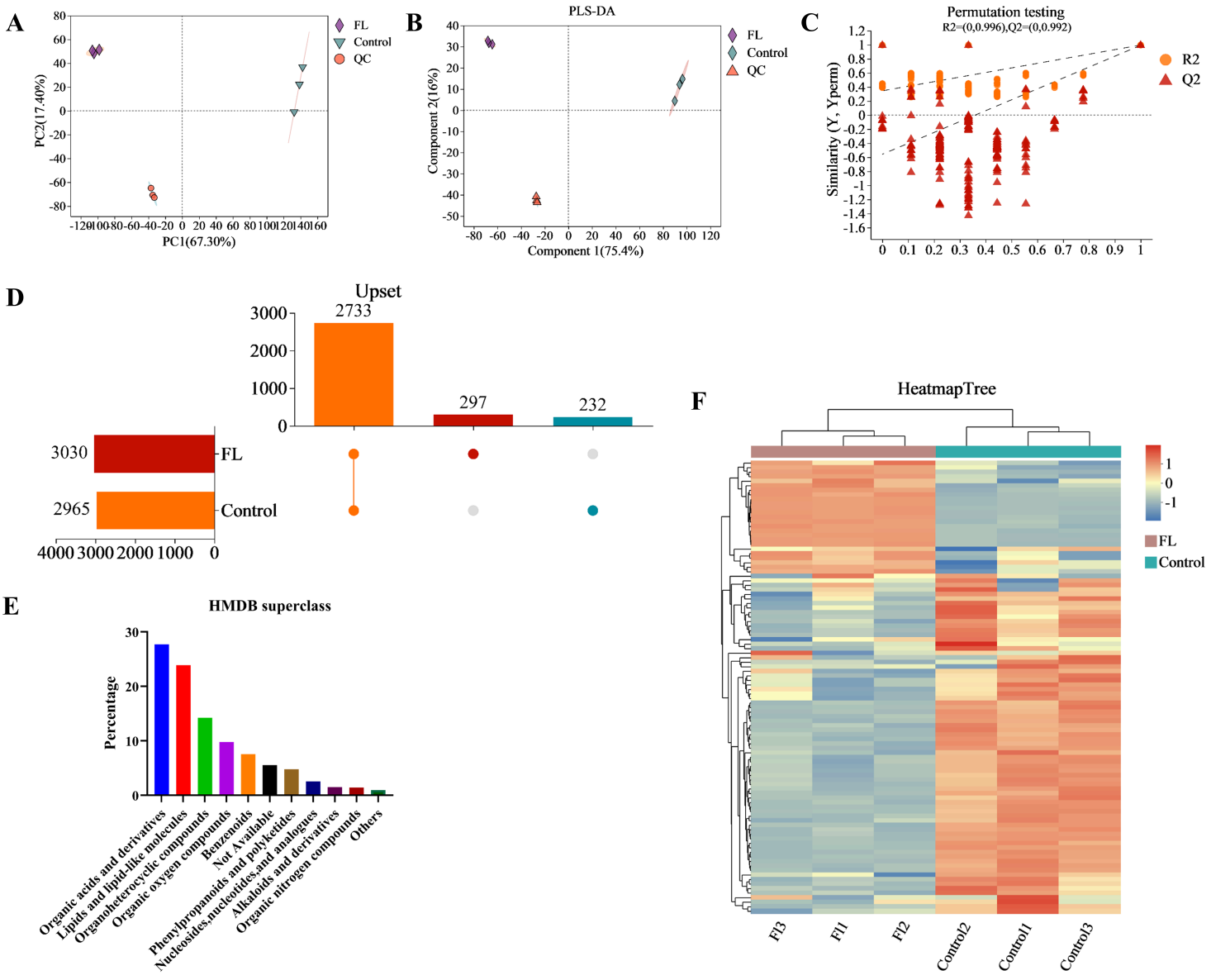


Figure 5

Comparative analysis of the samples. (A) PCA analysis. (B) PLS-DA analysis. (C) Permutation testing. (D) Upset. (E) HMDB compound classification. (F) Metabolite clustering analysis.

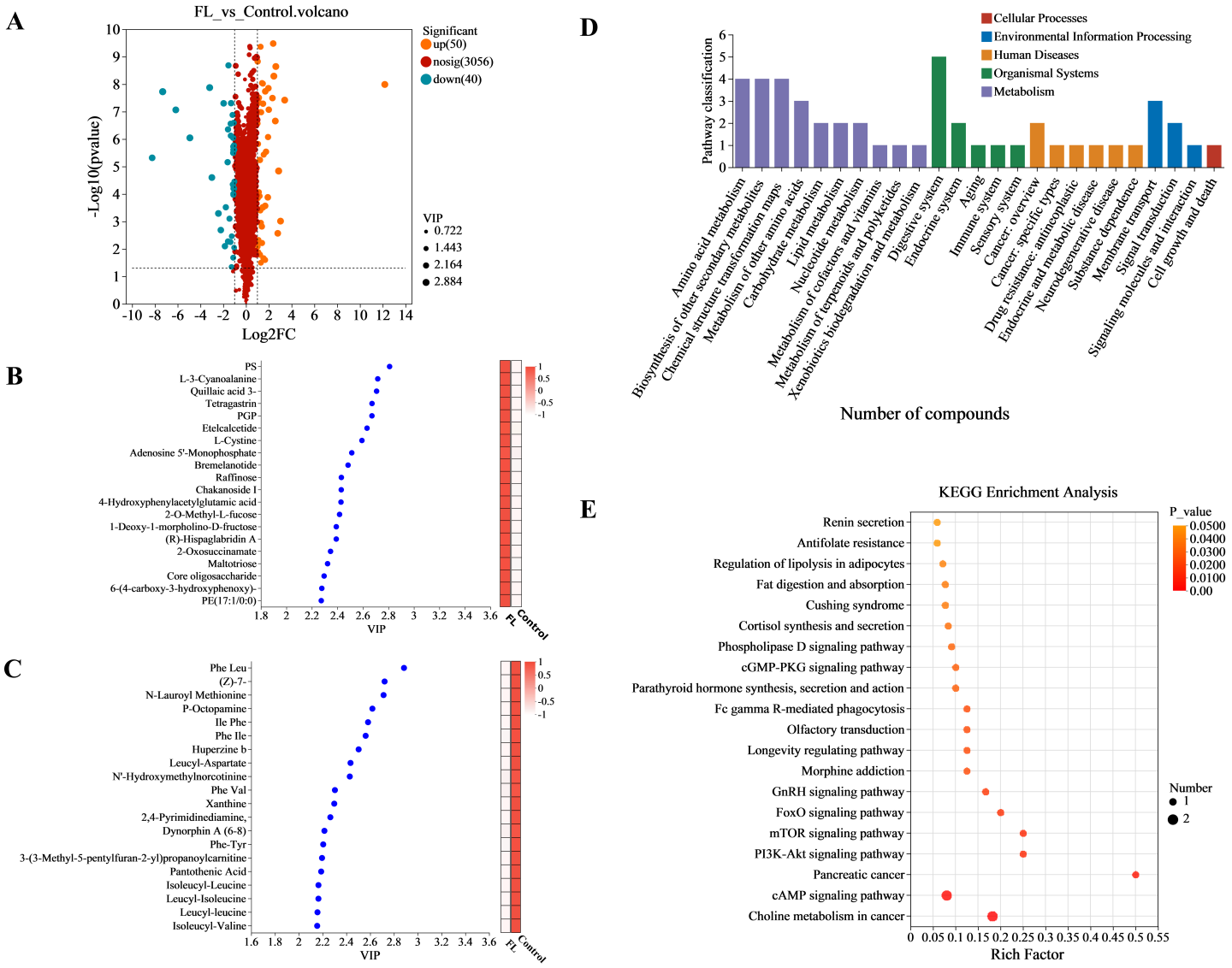


Figure 6

Analysis of differential metabolites. (A) Differential volcano map. (B) Up-regulated metabolite VIP score chart. (C) Down-regulated metabolite VIP score chart. (D) Analysis of KEGG functional pathways. (E) KEGG enrichment analysis.

Note: In figure B, PS: PS (20:4(5Z,8Z,11Z,14Z)/20:5(5Z,8Z,10E,14Z,17Z)-OH (12)). PGP: PGP (22:6(4Z,7Z,11E,13Z,15E,19Z)-2OH (10S,17)/i-15:0). 6-(4-carboxy-3-hydroxyphenoxy)-: 6-(4-carboxy-3-hydroxyphenoxy)-3,4,5-trihydroxyoxane-2-carboxylic acid. In figure C, 2,4-Pyrimidinediamine, :2,4-Pyrimidinediamine, 5-(5-(1-piperazinylmethyl)-1,3,4-oxadiazol-2-yl)-N4-propyl-N2-(2-(4-pyridinyl) ethyl)-.

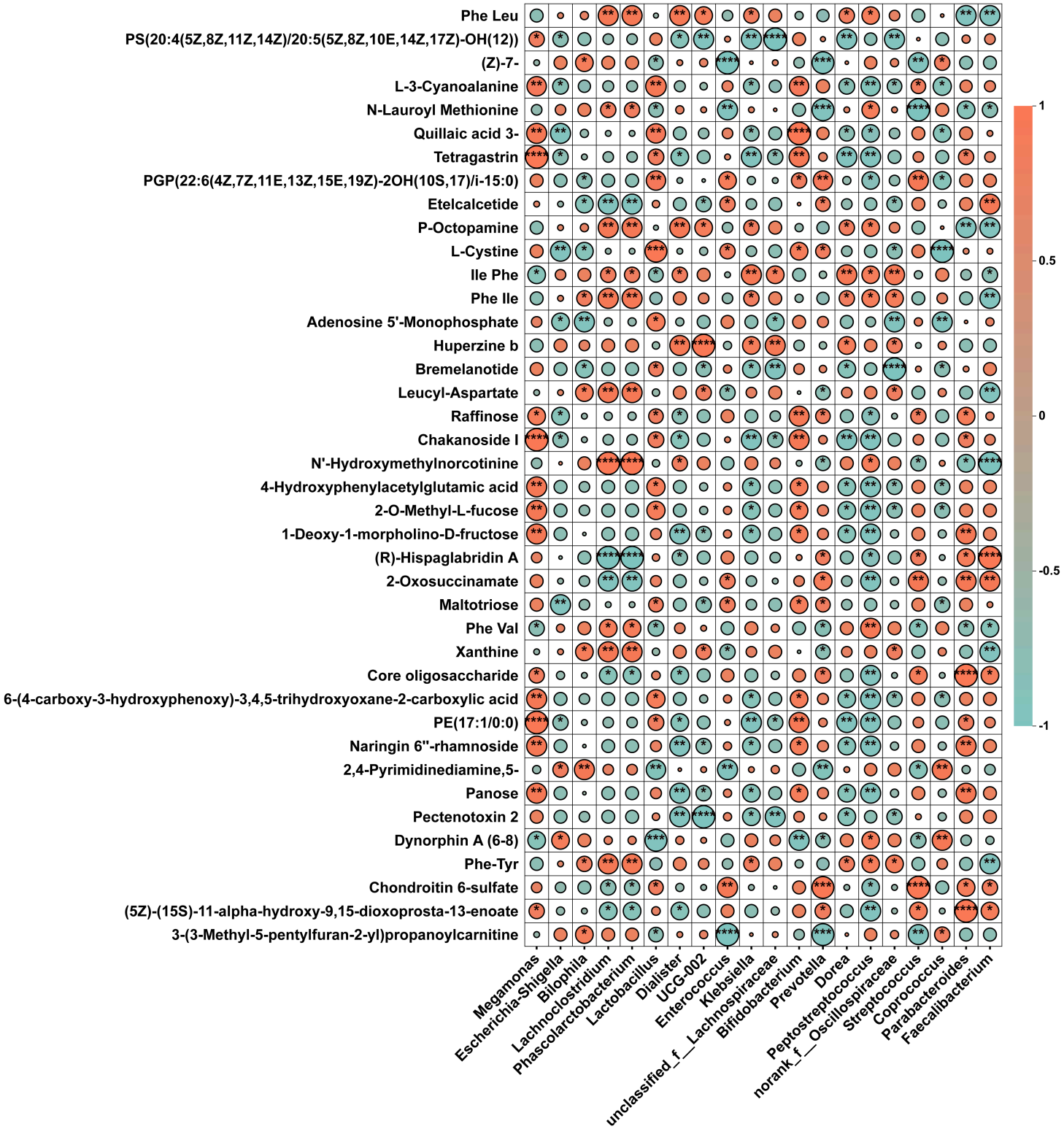


Figure 7

Correlation analysis between the gut microbiota and metabolites.