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Gut microbiota dysbiosis in rats with LPS-induced liver diseases affected by *Aronia melanocarpa* polyphenols

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Abstract

It is well known that there is an imbalance of gut microbiota in liver diseases, our previous study has proved that *Aronia melanocarpa* polyphenols (AMPs) can modulate the gut microbiota and affect the progression of liver diseases. Here, we analyzed the gut microbiota by 16S rRNA sequencing and bioinformatic analysis to explored the changes of gut microbiota composition and functions after LPS and AMPs intervention. Our results showed that there were significant differences in the gut microbiota structure between different treatment groups, such as increasing the abundance of *Lactobacillaceae* and *Muribaculaceae*, decreasing the abundance of *Ruminococcaceae* and *Acidaminococcaceae*. Furthermore, PICRUSt prediction showed that 29 functional pathways have changed significantly which may promote the treatment of liver diseases. This study could help to supplement the information about the community of gut microbiota in liver diseases and provide a new strategy for the treatment of liver diseases.

Keywords: *Aronia melanocarpa*; polyphenols; gut-liver axis; 16S rRNA sequencing; bioinformatic analysis.

Practical Application: Providing a theoretical basis for the prevention and treatment of liver diseases with natural functional foods, and of great significance for the in-depth development of Aronia melanocarpa.

1 Introduction

Hepatitis B virus (HBV), hepatitis C virus (HCV), alcoholic liver disease (ALD), nonalcoholic fatty liver disease (NAFLD), liver fibrosis, liver cirrhosis and hepatocellular carcinoma (HCC) are the most common liver diseases as we known (Liu & Jian, 2020). In the past period of time, although there have been many studies on liver diseases and great progress has been made in the treatment of liver diseases, liver disease is still one of the top ten causes of death in the world (Wang et al., 2020). About 300 million people in China suffer from liver diseases, and the number of patients with liver disease ranks the top in the world (Wang et al., 2015). Our previous study has shown that the possible mechanism of liver disease is mainly the imbalance of gut microbiota caused by LPS, lead the weakening of intestinal barrier function, then the increase of intestinal permeability caused the translocation of LPS and harmful bacteria into liver via portal circulation, and activating the inflammatory response (Kong et al., 2021). At present, the imbalance of gut microbiota is considered to be an important cause of liver diseases, which has attracted extensive attention of scholars all over the world.

In recent years, more and more studies have proved that there are many interactions between gut and liver, and the gut-liver axis plays a very important role in the occurrence and development of liver diseases. The gut-liver axis refers to the complex two-way communication between the gastrointestinal tract and the liver through the biliary tract, the portal vein, and systemic circulation (Han et al., 2023), 70% of the blood in the liver is provided by the portal vein system, the substances and their metabolites in the gut flow directly into the liver through the intestinal barrier, at the same time, the substances produced by the liver will be transmitted to the gut along with the biliary tract and systemic circulation, affecting the gut microbiota and intestinal barrier function (Jiang et al., 2019; Mohammadmoradi et al., 2014). 16S rRNA sequencing method is widely used to study the structure and composition of gut microbiota, feces material is a common substitute for gut microorganisms (Thomas et al., 2015). Current studies have shown that there are different degrees of gut microbiota dysbiosis in rats with liver diseases, for example, the abundance of gram-negative bacteria increased in rats with liver diseases induced by ethanol, leads to the increase of LPS content and then cause the liver diseases (Dong, 2020); The dysbiosis of gut microbiota in rats with liver disease induced by high-fat diet leads to the abnormality of bile acid metabolic pathway, which further aggravates the liver disease (Cheng et al., 2019). Therefore, liver diseases are largely caused by the dysbiosis of gut microbiota, targeting the regulation of gut microbiota may be an effective method to treat liver diseases.

Aronia melanocarpa (black chokeberry), a native shrub in eastern North America, belongs to the genus *Aronia* (Rosaceae family, Maloideae subfamil), is very popular in eastern Europe and Russia, was usually used to treat colds in the 20th century, at present, *A. melanocarpa* is mainly used for drinks production, natural pigments extraction and ornamental plants (Kokotkiewicz et al., 2010; Valcheva-Kuzmanova & Belcheva, 2006). *A. melanocarpa* fruits are one of the richest plant sources of polyphenols (Valcheva-Kuzmanova & Belcheva, 2006), and has a variety of health promotion characteristics on chronic

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diseases, such as anti-oxidation, anti-tumor, anti-inflammatory and anti-aging, protecting gastrointestinal and liver systems (Platonova et al., 2021; Kokotkiewicz et al., 2010). Our previous studies have also proved that *A. melanocarpa* has the functions of preventing Alzheimer's disease (Meng et al., 2018) and antibacterial properties (Deng et al., 2021).

Polyphenols are important secondary metabolites of plants, mainly including anthocyanins, flavonols, and hydroxycinnamates and other bioactive substances, therefore, they have very strong antioxidant function and are the most abundant antioxidants in human diet (Xie & Zou, 2013). At the same time, as a substance that can promote the colonization of beneficial gut bacteria and inhibit harmful or pathogenic bacteria, polyphenols can regulate the structure and composition of gut microbiota (Naggar & Wang, 2021), play an immunomodulatory role and inhibit the pathogenesis of various diseases, and have good therapeutic effects on liver injury (Kong et al., 2021), colitis (Tan et al., 2021), obesity (Jiao et al., 2019) and other chronic diseases. For example, Xia et al. (2021) showed that after the intervention of polyphenols extract from plants, the imbalance of gut microbiota in rats with was improved, thus reduced liver inflammation and prevented liver disease. Luo et al. (2021) found that polyphenols extracted from honey can improve the ratio of *Firmicutes* and *Bacteroides*, and prevent liver disease. However, the effect of polyphenol extract of *A. melanocarpa* on gut microbiota and its mechanism on LPS-induced liver diseases remain unclear.

Therefore, the purpose of this study was to elucidate the effect of *A. melanocarpa* polyphenols (AMPs) on the gut microbiota of rats with LPS-induced liver diseases, and to characterize the gut microbiota of rats with LPS-induced liver diseases by high-throughput 16S rRNA sequencing. The purpose of this study was to analyze the differences of gut microbiota between pre-polyphenol and post-polyphenol intervention, indicating that polyphenols may be used as a gut microbiota regulator and could mediate gut-liver axis, to investigate the effect of polyphenol treatment on gut microbiota in rats with LPS-induced liver diseases, thereby providing a theoretical basis for further revealing the preventive mechanism of gut microbiota on the occurrence and development of liver diseases and providing a new target for the treatment of liver diseases.

2 Material and methods

2.1 Chemicals and reagents

A. melanocarpa (Fu Kangyuan No.1, black chokeberry) were obtained from Fu Kangyuan Black Chokeberry Technology Co., Ltd. (Haicheng, Liaoning). 30 Sprague-Dawley male rats were obtained from Wanlei Bio Co., Ltd., Shenyang, China Ltd (Beijing, China). *A. melanocarpa* polyphenols (AMPs) were extracted and purified in the Fruit and Vegetable Processing Laboratory, Shenyang Agriculture University, Liaoning, China. The reagents needed for fecal DNA extracted were DNA isolation kit, (Tiangen BioTeke, Beijing, China). The reagents needed for PCR products quantification and qualification were Phusion High-Fidelity PCR Master Mix (M0531S, New England Biolabs), High-Fidelity DNA polymerase (Phusion M0530S, New England Biolabs), forward primers and reverse primers, and ddH2O, PCR product purification kit were Universal DNA kit (TianGen, China), sequencing libraries were generated with NEB Next Ultra DNA Library Prep Kit (Illumina, San Diego, CA, USA).

2.2 Preparation of A. melanocarpa polyphenols (AMPs)

The extraction and purification method were performed as we reported previously (Kong et al., 2021). Briefly, *A. melanocarpa* (10 g) were extracted with ultrasonic assisted extraction in an 55% ethanol aqueous solution (440 mL) for 40 minutes. Use D-101 macroporous resin (D-101; Dingguo, Shanghai, China) and a rotary evaporator (XD-5210A; Rotary Evaporator, Xiande Instrument Co., Ltd., Shanghai, China) to purified the extract. Finally, the freeze-drying treatment was carried out and the extract was stored at -20 °C. According to the analysis of HPLC, the main content of anthocyanins in AMPs was about 2255.00 μg/mL, contained cyanidin-3-O-galactoside, cyanidin-3-O-glucoside, cyanidin-3-O-arabinoside, and cyanidin-3-O-xyloside; flavonol was about 52.00 μg/mL, contained quercetin-3-O-nestinoside, quercetin-3-O-robinobioside, quercetin-3-O-rutinoside, quercetin-3-O-galactoside, and quercetin-3-O-glucoside; hydroxycinnamate was about 432.00 μg/mL, contained chlorogenic acid, neochlorogenic acid, and caffeic acid.

2.3 Animal treatments

All experiments were conducted according to the Ethics Committee of Shenyang Agricultural University (2022051304). Animal treatment were consistent with our previous study (Kong et al., 2021). Eight weeks old male Sprague-Dawley rats (210-230 g) were obtained from Wanlei Bio Co., Ltd. (Shenyang, China). After adapted to the environment (temperature 25 °C ± 2 °C, 60% humidity, 12 h circadian cycle) for 1 week, during this time the rats fed diet (54% carbohydrate, 18% protein, 4% fat and 5% fiber) and drank water freely (Mu et al., 2020).

Rats were randomly divided into five groups (n = 6), based on the criteria from the previous studies (Kong et al., 2021). All treatments were administered by gavage every day. After 4 weeks, all rats were executed with pentobarbital sodium (200 mg/kg BW) after the last feeding for 12 hours. In an aseptic environment, fresh feces of rats were collected for analysis and rapidly frozen and storage at -80 °C in liquid nitrogen. The diet compositions of different groups were shown in Table 1.

Table 1. Diet compositions of different groups.

Group	Diet
control group (CG)	distilled water
model group (MG)	LPS (200 μg/kg BW)
low polyphenol dose group (LP)	LPS (200 μg/kg BW) + AMPs (50 mg/kg BW)
middle polyphenol dose group (MP)	LPS (200 μg/kg BW) + AMPs (100 mg/kg BW)
high polyphenol dose group (HP)	LPS (200 μg/kg BW) + AMPs (200 mg/kg BW)

2.4 DNA extraction

DNA was extracted by using the DNA isolation kit according to the manufacturer's instructions. Add 0.25 g fecal samples into 2ml centrifuge tube, mixed with 500 μl buffer SA, 100 μl buffer SC, 0.25 g grinding beads and 10 μl RNase A by vortex vibration, and then heat the mixture at 70 °C for 15 min. Centrifuge at 12000 rpm for 1 min. Transfer the supernatant to a 2 mL centrifuge tube with 200 μL buffer SH and centrifuge at 12000rpm for 3 mins, transfer the supernatant to a 2 mL centrifuge tube with 500 μl GFA. Add 10 μl magnetic bead suspension G mixed for 5 mins, add 700 μL deproteinized solution RD and 700 μL rinse solution PWD for magnetic bead adsorption, add 50 μL elution buffer TB, after the magnetic beads were adsorbed, stored the DNA solution for the follow-up test.

2.5 Amplicon generation

Fecal DNA was used as a template for the PCR amplification and the bacterial universal primers of 338F (5'-ACTCCTACGGGAGGCAGCA-3) and 806R (5'-GGACTACHVGGGTWTCTAAT-3) were used. The PCR reactions were carried out with 15 μL of Phusion High-Fidelity PCR Master Mix, 1 μM of each primer and 10ng target DNA 0.2 μM of forward and revers primers. Thermal cycling consisted of initial denaturation at 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C, 10 s; 50 °C, 30 s; 72 °C, 30 s; 72 °C, 5 min.

2.6 PCR products quantification and qualification

The buffer containing SYB green was mixed with PCR products in a volume ratio of 1:1, and DNA was detected by agarose gel electrophoresis containing 2%. Mix the PCR products in equal proportion, and finally purify the mixed PCR products with DNA-PCR purification kit according to the manufacturer's instructions.

2.7 16S rRNA library preparation and sequencing

The library was sequenced on an Illumina NovaSeq platform, the library quality was assessed on the Agilent Bioanalyzer 2100 system and quantified by real-time PCR (1.5 Nm), then 250 bp paired-end reads were generated. Row tags were merged using FLASH (V1.2.7), using QIIME (V1.9.1) to obtain high-quality clean tags by quality filtering on the raw tags, the splicing sequences were called raw tags. Use UCHIME algorithm to detect chimera sequences, and removed the chimera sequences to get the effective tags.

2.8 Bioinformatics analysis.

OUT analysis

Sequences analysis were performed by Uparse software (v7.0.1001). The sequences with similarity 97% were grouped into one OTUs. The representative sequences of each OTU were screened and annotation. In order to evaluate the number of shared OTU's between the samples, the data of each sample should be homogenized, subsequent analysis of alpha diversity and beta diversity were all performed basing on this output normalized data. A Venn diagram was constructed using Mothur and SSUrRNA database of SILVA132.

Alpha diversity

ACE and Simpson indices were assessmented with QIIME (Version 1.7.0) and use R software (version 2.15.3) to draw the rank abundance curve. UniFrac distance and Unweighted Pair-group Method with Arithmetic Mean (UPGMA) sample cluster tree was calculated and constructed by Qiime software (version 1.9.1).

Beta diversity

Principal Coordinate Analysis (PCoA) and non-metric multidimensional scaling (NMDS) were performed to examine sample clustering by reducing the dimension of the original variables, the weighted or unweighted UniFrac distance matrix between the samples obtained before is transformed into a new set of orthogonal axes, in which the maximum change factor is represented by the first principal coordinate, the second maximum change factor is represented by the second principal coordinate. Using the WGCNA, stats, ggplot2 and vegan packages in R software (Version 2.15.3) to analysis and drawn PCoA, NMDS diagrams.

PICRUSt prediction

Functional genes were predicted using PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states) with the KEGG (Kyoto Encyclopedia of Genes and Genomes) database based on the OTU tree of Greengene database and the gene information on OTU.

2.9 Statistical analysis

R software (version 2.15.3) and QIIME software (1.9.1) were used for statistical analysis. T-test and Tukey multiple comparison analysis was performed to compare categorical and continuous variables between groups. p values < 0.05 or < 0.01 were considered statistically significant.

3 Results

3.1 Venn

At present, in the study of gut microbiota based on 16S rRNA gene, 97% sequence similarity is usually used as an OTU division threshold. In order to analyze the common and out of each group, we drew a Venn diagram (Figure 1A). Each ellipse represents a group of samples. The overlapping area between ellipses was common, and the none overlapping area was unique out; the number indicated the number of OTU. In this study, 1023, 1058, 987, 971, 970 OTUs were identified in the CG and MG, LP, MP, HP respectively. The Venn diagram displaying the overlaps between groups showed that among the total abundance of 5009 OTUs, 767 OTUs were shared among these five groups. It was worth noting that 26, 34, 24, 18, 7 OTUs were unique in CG, MG, LP, MP, HP and the abundance was low.

3.2 Alpha diversity analysis

The rank abundance curve, based on a 97% sequence similarity cutoff, can intuitively show the number of high abundance and rare OTU in the gut microbiota, implied that a majority of the bacterial OTUs were considered minor (relative abundance < 1%), the results were shown in Figure 1B. Consistent with the Venn diagram, the number of OTU in MG is the highest. After AMPs intervention, the number of OTU shows a downward trend compared with MG. In order to evaluate alpha diversity, ACE and simpson index results were shown in Figure 2. It was observed that MG increased species richness but not significantly compared with CG. After supplement of AMPs, AMPs group reduced species richness (ACE), LP, HP group were significantly decreased ($P < 0.01$, $P < 0.05$) compared with MG; Simpson index and ACE index had the same trend. Compared with CG, MG increased species diversity (simpson). According to the analysis of relative abundance of species, the increase of species diversity and abundance may be caused by the abundance of beneficial bacteria *Lactobacillus* increased, indicating that AMPs can improve the gut microbiota imbalance. On the contrary,

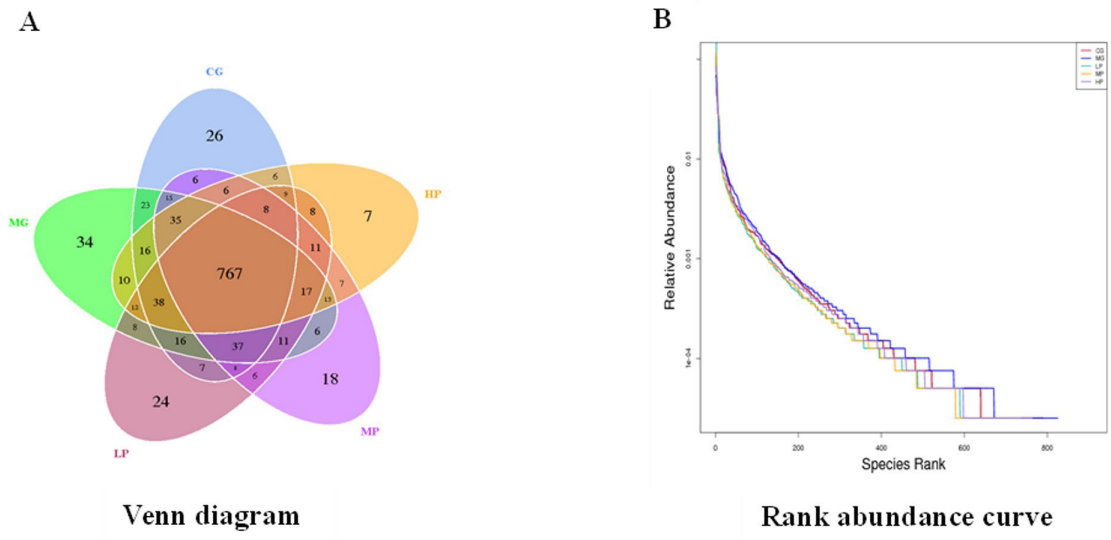

Figure 1. (A) Venn diagram of comparison of OUTs distribution based on normalized sequences and 97% sequence similarity among CG, MG, LP, MP, HP groups; (B) Rank abundance curve.

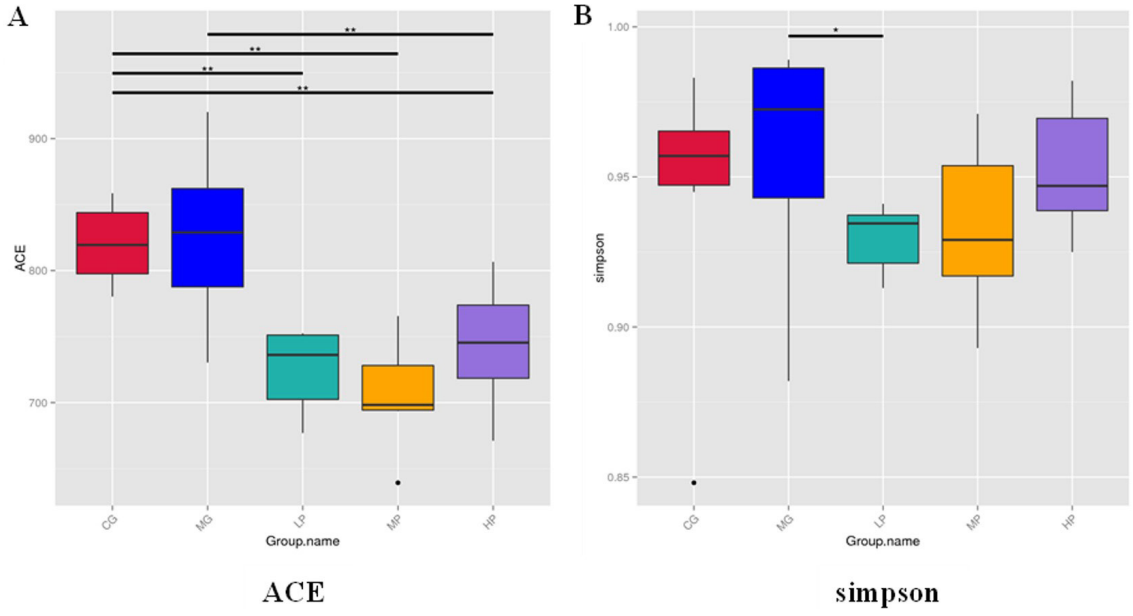

Figure 2. Comparison of alpha diversity accessed by ACE and Simpson index. (A) ACE; (B) Simpson.

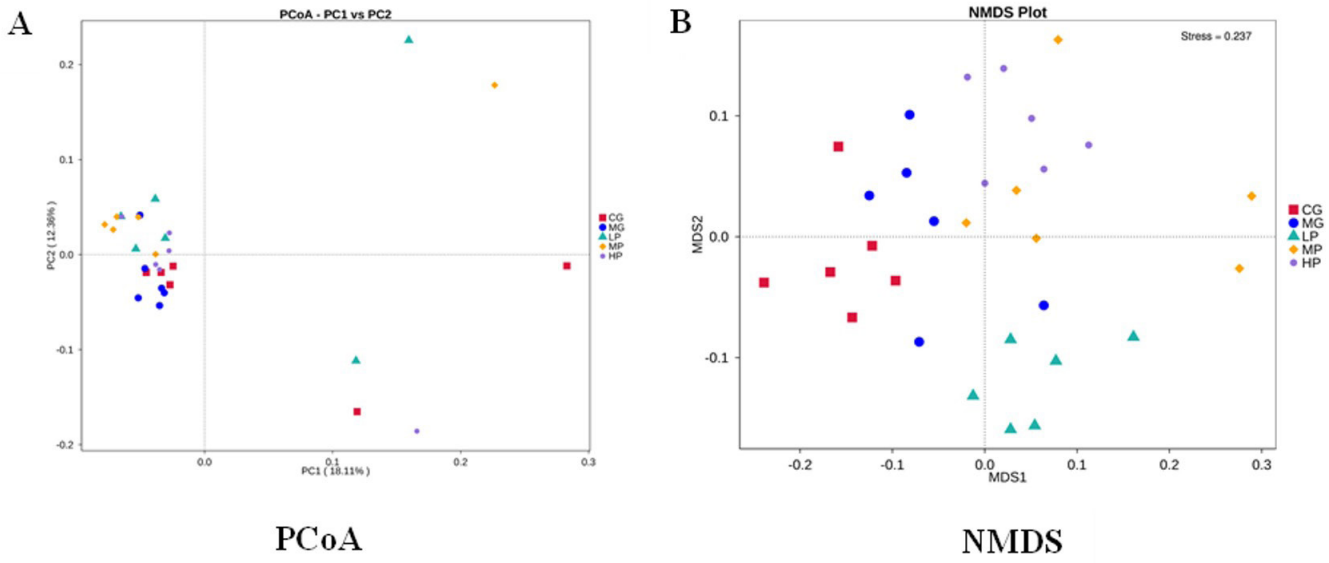

Figure 3. (A) The results of PCoA determined by an unweighted UniFrac principal coordinate analysis based on OTU composition; (B) The results of NMDS of gut microbiota communities.

compared with MG, the three AMPs groups decreased species diversity, but there was no significant difference between these groups, which was consistent with our previous research.

3.3 Beta diversity analysis

In order to evaluate beta diversity, unweighted UniFrac distance-based principal coordinate analysis (PCoA), and non-metric multidimensional scaling (NMDS) based on the unweighted UniFrac distances between the fecal samples were conducted were shown in Figure 3. PCoA and NMDS are based on UniFrac distance, which can more comprehensively reflect the similarity between community samples by comparing the phylogenetic relationship between samples OTU. The results of PCoA showed that the first three components explained 39.06% of the total variance (PC1, PC2 and PC3 were 18.11%, 12.36% and 8.59% respectively). CG, MG and AMPs treatment groups had separate unique microbiota, LP, MP and HP were intermingled and formed overlapping clusters, which were both similarity and variance. MG were slightly different from CG. NMDS analysis produced similar results, the five groups had separate unique microbiota.

3.4 OTU cluster abundance analysis

To determine the structural changes of the gut microbiota, the microbial composition was compared at different classification levels. At the class level, *Clostridia*, *Bacilli*, *Bacteroidia*, *Erysipelotrichia*, *Negativicutes*, *Gammaproteobacteria*, *unidentified_Actinobacteria*, *Deltaproteobacteria*, *unidentified_Bacteria*, *Coriobacteriia* were the top ten class in the gut microbiota. The dominant class were *Clostridia*, *Bacilli*, *Bacteroidia*, accounting for more than 50% of the class. The proportion of dominant class in each group is shown in Figure 4A. At the class level, the MG had significantly ($p < 0.01$) increased abundance of *Clostridia* and *Negativicutes*

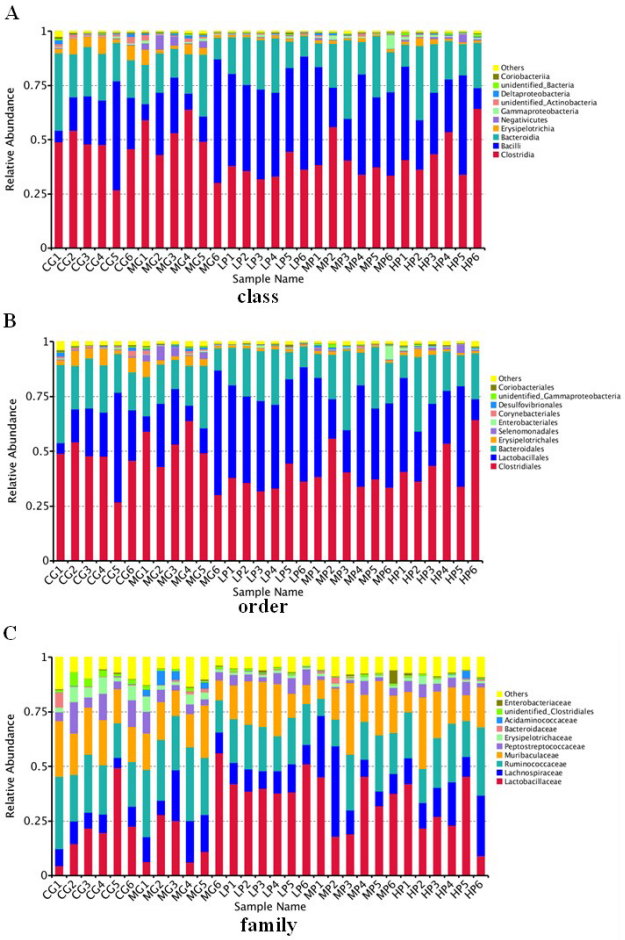

Figure 4. Effects of AMPS on gut microbiota composition. (A) Microbial composition at class level; (B) Microbial composition at order level; (C) Microbial composition at family level.

while the abundance of *Bacteroidia* were significantly ($p < 0.01$) decreased. After AMPs supplementation, when compared with MG, the relative abundance of *Bacilli*, *Bacteroidia* and *Gammaproteobacteria* was significantly ($p < 0.01$) increased in the AMPs group, whereas that of *Clostridia*, and *Negativicutes* was significantly decreased in the AMPs group.

At the order level, *Clostridiales*, *Lactobacillales*, *Bacteroidales*, *Erysipelotrichales*, *Selenomonadales*, *Enterobacteriales*, *Corynebacteriales*, *Desulfovibrionales*, *unidentified_Gammaproteobacteria*, *Coriobacteriales* were the top ten order in the gut microbiota. The dominant class were *Clostridiales*, *Lactobacillales*, *Bacteroidales*, accounting for more than 90% of the order. The proportion of dominant class in each group is shown in Figure 4B. At the order level, the MG had significantly ($p < 0.01$) increased abundance of *Clostridiales*, Selenomonadales, Enterobacteriales, and *unidentified_Gammaproteobacteria* while the abundance of *Bacteroidales*, *Erysipelotrichales*, *Corynebacteriales*, *Desulfovibrionales*, *Coriobacteriales* were significantly ($p < 0.01$) decreased. After AMPs supplementation, when compared with MG, the relative abundance of *Lactobacillales*, *Enterobacteriales* and *Gammaproteobacteria* was significantly ($p < 0.01$) increased in the AMPs group, whereas that of *Clostridiales*, *Selenomonadales*, was significantly decreased in the AMPs group.

At the family level, *Lactobacillaceae*, *Lachnospiraceae*, *Ruminococcaceae*, *Muribaculaceae*, *Peptostreptococcaceae*, *Erysipelotrichaceae*, *Bacteroidaceae*, *Acidaminococcaceae*, *unidentified_Clostridiales, Enterobacteriaceae* were the top ten order in the gut microbiota. The dominant family were *Lactobacillaceae*, *Lachnospiraceae*, *Ruminococcaceae*, *Muribaculaceae*, accounting for more than 70% of the family. The proportion of dominant class in each group is shown in Figure 4C. At the family level, the MG had significantly ($p < 0.01$) increased abundance of *Lachnospiraceae*, *Ruminococcaceae*, *Acidaminococcaceae* and while the abundance of *Muribaculaceae*, *Peptostreptococcaceae*, were significantly ($p < 0.01$) decreased. After AMPs supplementation, when compared with MG, the relative abundance *Lactobacillaceae* and *Muribaculaceae* was significantly ($p < 0.01$) increased in the AMPs group, whereas that of *Ruminococcaceae* and *Acidaminococcaceae* was significantly decreased in the AMPs group.

The clustering heat map generated from the species richness of the first 20 species at the class level, the first 35 species at the order and family level are shown in Figure 5. Compared with the CG, the MG had different gut microbiota composition, which was modified by AMPs supplementation but remained different from the CG.

3.5 Phylogenetic Unvestigation of Communities by Reconstruction of Unobserved States (PICRUSt) analyses

We transformed the composition of OTUs sequence into the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthogonality and generated KEGG map to analyze the differences of metabolic pathways and functions of gut microbiota after LPS and AMPs intervention in rats (Figure 6). Through PICRUSt analysis, a total of 39 metabolic functions were predicted in all samples at level 2, above all, the functions of gut microbiota were mainly concentrated in membrane transport (13.11%); carbohydrate metabolism (10.80%); amino acid metabolism (9.24%); replication and repair (9.17%) and translation (5.94%).

There were 328 KEGG functional pathways at level 3, among them, compared with CG group, MG group enriched 5 functional pathways ($p < 0.05$), including cytoskeleton proteins, protein kinases, carbohydrate metabolism, lipid metabolism and biosynthesis of unsaturated fatty acid, the representation of 3 functional pathways in MG group were less represented in CG group ($p < 0.05$), including peptidases, citrate cycle (TCA cycle) and tryptophan metabolism. After AMPs intervention, compared with MG group, LP group was enriched in 5 pathways, ABC transporters, two-component system, sporulation, plant-pathogen interaction and biosynthesis of ansamycins. The representativeness of 6 pathways in LP was lower than that in MG group ($P < 0.05$), including peptidase pyrimidine metabolism, galactose metabolism, amino acid metabolism, D-glutamine and D-glutamate metabolism, ubiquinone and other terpenoid-quinone biosynthesis and taurine and hypotaurine metabolism. Compared with MG group, MP group enriched 4 pathways, these were bacterial motility proteins, bacterial chemotaxis, flagella assembly, flagella assembly and plant-pathogen interaction ($P < 0.05$), the expression of 5 pathways decreased ($P < 0.05$), including chaperones and folding catalysts, cell cycle-caulobacter, nicotinate and nicotinamide metabolism, membrane and intracellular structural molecules and folate biosynthesis. Compared with MG group, HP group was enriched with 1 functional pathway ($P < 0.05$), which was galactose metabolism.

4 Discussion

LPS-induced liver disease in rats, which is a liver injury related to the imbalance of gut microbiota. The genetic material contained in the gut microbiota is several times that of human body and has many function characteristics. It affects human physiological health by producing countless metabolites, peptides and other substances. The liver is the link of connecting nutrition and toxin sources between other parts of human body. LPS is the main component of the outer wall of the cell membrane of gram-negative bacteria, which may lead to the imbalance of gut microbiota, and can also pass through the intestinal barrier to enter the blood circulation of the body, activate inflammatory cytokines and activate the inflammatory signaling pathways to induce liver inflammatory reaction, cause cell apoptosis and liver damage. In recent years, the composition and function of gut microbiota play a vital role in human diseases, gut-liver axis is widely considered to be related to the pathogenesis of liver diseases (Wiest et al., 2017). As a natural plant bioactive substance, polyphenols have attracted more and more attention. Some studies have shown that polyphenols can alleviate liver diseases and promote human health by modulating the gut microbiota structure (Lu et al., 2021). Different sources of polyphenols may change different bacterial populations, but they all increased the abundance of beneficial bacteria and reduced the abundance of harmful bacteria, thus alleviating the body damage. Our previous studies have also proved that *A. melanocarpa* polyphenols (AMPs) can modulate the gut microbiota to improve LPS-induced liver

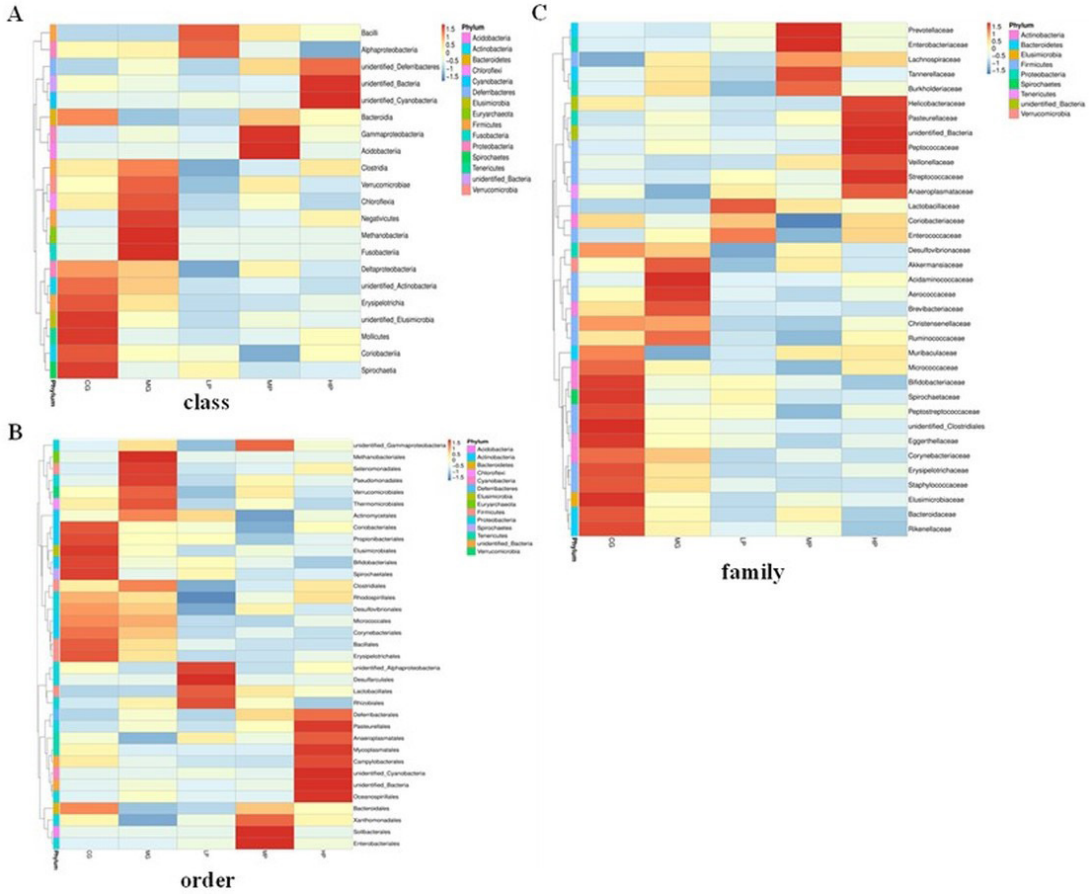

Figure 5. Cluster heat map of species richness at different levels. (A) class level; (B) order level; (C) family level. [[Q4: Q4]]

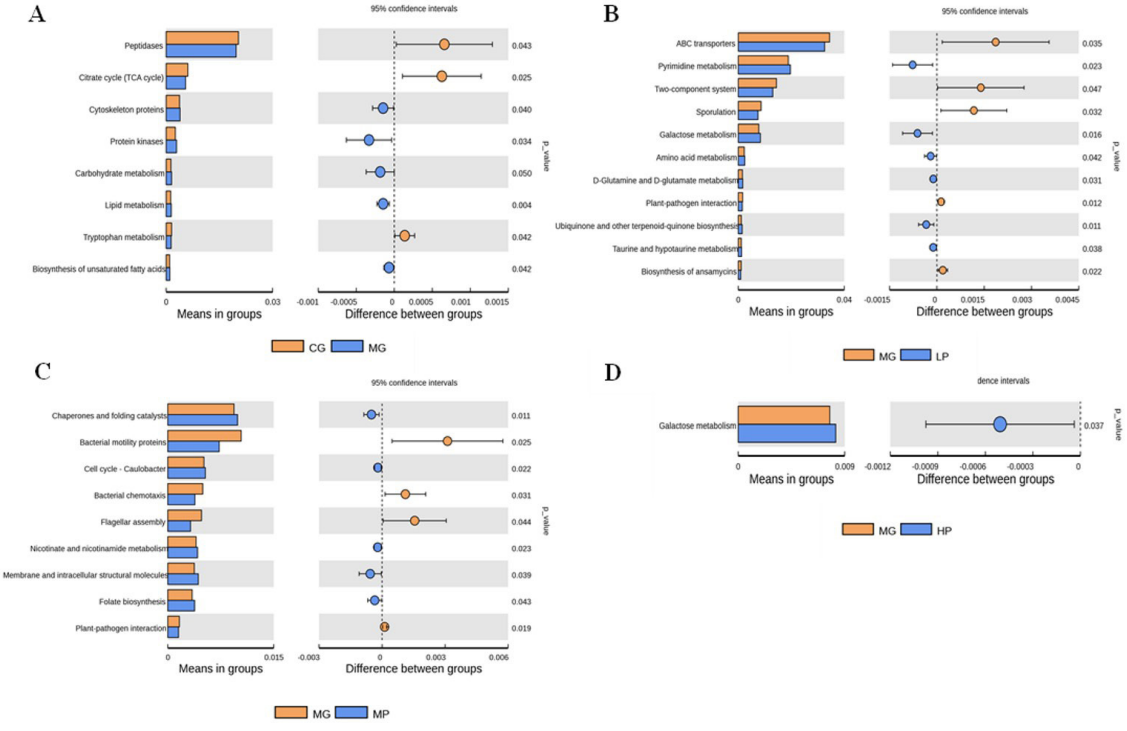

Figure 6. Alternation of predicted microbial functional composition from 16s rRNA sequencing data with PICRUSt. (A) CG vs MG; (B) MG vs LP; (C) MG vs MP; (D) MG vs HP.

diseases in rats and protect the liver, and in a dose-dependent manner (Kong et al., 2021). However, it is still unclear how the *A. melanocarpa* polyphenols (AMPs) can modulate the composition and function in the gut microbiota of rats with LPS-induced liver diseases. Therefore, in this study, 16S rRNA gene sequencing was used to analyze the gut microbiota of rats with LPS-induced liver diseases, in order to discuss how *A. melanocarpa* polyphenols (AMPs) modulate the composition and function of gut microbiota in LPS-induced liver diseases of rats, so as to analyze the possibility of prevention of liver diseases.

Previous studies have shown that diet could modulate the gut microbiota composition and metabolism, marking gut microbiota a relationship between diet and different physiological index through their capacity to generate microbial composition and metabolites due to the dietary intake (Zheng et al., 2020). Our study showed that LPS and AMPs treatment did change the composition of gut microbiota at various classification levels, including *Clostridia*, *Bacteroidia*, *Lactobacillales*, *Selenomonadales*, *Lachnospiraceae*, *Ruminococcaceae*, etc. Wang et al. (2021b) discussed that polyphenols control liver diseases through gut-brain-liver axis, the possible mechanism involved the change of gut microbiota composition, Wu et al. (2018) also proved that *Lonicera caerulea L.* polyphenols can reduce the inflammatory response of liver diseases by modulating the gut microbiota especially changing the ratio of beneficial bacteria to harmful bacteria, which were consistent with our research results.

Venn diagram shows the number of similar gut microbiota or endemic gut microbiota between different groups. It can be inferred from the results of this study that the number of OTUs in the MG increased slightly compared with CG, and the number of endemic gut microbiota decreased compared with MG after AMPs intervention. Indicating that different treatment groups have different active populations. This difference may be the result of different feeding conditions in each treatment groups. It can be inferred that the abundance of harmful bacteria or conditional pathogenic bacteria in the MG increased, and the number of harmful bacteria or conditional pathogenic bacteria decreased after AMPs intervention. With the increase of dose, the number of harmful bacteria or conditional pathogenic bacteria was lower than the CG, it can be inferred that AMPs can also prevent and regulate the imbalance of gut microbiota.

ACE and Simpson index showed the richness and the diversity of gut microbiota. The results showed that the richness and diversity of gut microbiota in MG are higher than those in CG, which may due to the imbalance of gut microbiota in LPS-induced liver diseases in rats, LPS may cause the abundance of harmful bacteria or conditional pathogens in MG increased. After AMPs intervention, the richness and diversity of gut microbiota were lower than those in CG, it played a positive regulatory role, indicating that the composition of gut microbiota is closely related to the LPS-induced liver diseases, and AMPs may play a role of probiotics and prevent gut microbiota imbalance. The results were consistent with the results of Venn diagram.

These results of Beta diversity analysis showed that the microbial community structure of MG group is different from that of CG group, indicated that LPS may change the structure of gut microbiota and lead the disorder of the gut microbiota. The microbiota community structure of the three AMPs groups is similar, but different from that of CG and MG groups, indicated that after supplemented with AMPs, the gut microbiota structure of LPS-induced rats is similar, it can be inferred that AMPs supplementation can improve the imbalance of gut microbiota in rats with liver diseases induced by LPS, and AMPs may have a positive regulatory effect on normal gut microbiota. This provides a basis for further study on the effect of polyphenols on gut microbiota.

In order to study the effect of AMPs on the composition of gut microbiota, we mainly analyzed the changes of different bacterial abundances at the family level, and found that the aboudance of *Lachnospiraceae*, *Ruminococcaceae*, *Muribaculaceae*, *Peptostreptococcaceae* and *Actobacillaceae* changed significantly. It has been proved that *Lachnospiraceae* is a secondary colonic bile acids (BAs) producing bacteria, and the level of colonic secondary bile acids (BAs) related bacteria is positively correlated with liver inflammation and liver diseases (Hz et al., 2020). Lee, G. et. al found that the aboundance of *Ruminococcaceae* was positively correlated with the degree of liver fibrosis in non-obese examinees (Lee et al., 2020). But some study showed that the aboundance of *Ruminococcaceae* was decreased in patients with non-alcoholic steatohepatitis (NASH), which was different from our results, this research also pointed out that this difference may be related to the external conditions such as regions (Tsai et al., 2020). Yuan et al. (2021) found that dietary intervention could improve the liver diseases induced by high-fat diet by resisting the decrease of *Muribaculaceae* abundance. Study showed that 16S rRNA sequencing of whole colon homogenate containing feces showed a decrease in the abundance of *Peptostreptococcaceae* in LPS-induced liver diseases (Matsushita et al., 2016), but it was found that the abundance of *Peptostreptococcaceae* increased in the study of gut microbiota in rats with liver injury induced by high-fat diet (Dongmin et al., 2019). This may provide different therapeutic targets for liver diseases induced by different causes, these were consistent with our research. The cluster heat maps of species richness at different levels also showed that the gut microbiota composition had changed greatly between different treatment groups. These results showed that AMPs could modulate the imbalance of gut microbiota in rats of LPS-induced liver diseases.

Most polyphenols cannot be absorbed by the small intestine and will enter the colon to affect the biological activity of gut microbiota (Manach et al., 2004). The liver receives blood from the intestine and other organs, and as a link between the host and gut microbiota, it plays an important role in the metabolic pathway of gut microbiota. When the metabolism of intestinal microflora is abnormal, it will stimulate malignant changes in the liver, such as inflammation (MacPherson et al., 2016). Therefore, when there is a liver disease, the metabolic pathways of gut microbiota will also be changed. Our results showed that there were differences in multiple metabolic pathways in different treatment groups, including 11 metabolic related pathways, including carbohydrate metabolism, lipid metabolism, citric acid cycle (TCA cycle), tryptophan metabolism, peptidase metabolism, galactose metabolism, amino acid metabolism, D-glutamine and D-glutamate metabolism, taurine and hypotaurine metabolism, nicotinic acid and nicotinamide metabolism

and galactose metabolism. Previous studies have shown that in nonalcoholic fatty liver disease (NAFLD), abnormal lipid metabolism of gut microbiota is conducive to steatosis and has a direct impact on the liver (Marra & Svegliati-Baroni, 2017). Michail et al. (2015) performed 16S rRNA gene sequencing, metagenomic shotgun sequencing, mass spectroscopy for proteomics and NMR spectroscopy for metabolite analysis on gut microbiota in children with liver diseases, proved that the metabolic pathways of carbohydrates and amino acids had changed, caused the energy metabolism was not provided by carbohydrates and amino acids metabolism, which led to the further development of the liver disease. Citrate cycle (TCA cycle) is the hub of energy metabolism, connecting a variety of metabolic pathways, it is a very important and ubiquitous metabolic pathway in aerobic organisms (Peng et al., 2019). Cheng et al. (2020) demonstrated that the anthocyanins in polyphenols can significantly regulate the structure of gut microbiota and regulate the KEGG pathway of microbial citrate cycle (TCA cycle) by improving key proteins. The level of nicotinamide metabolism can also change the antioxidant defense ability of inflammatory tissues (Peng et al., 2019).

It can be inferred that gut microbiota may affect LPS-induced liver disease in rats by regulating the different functional metabolic pathways. However, the data based on 16S rRNA gene sequencing is not comprehensive, it cannot identify the bacteria at the species level, nor can it detect nonbacterial microorganisms such as viruses and fungi in fecal samples, it can only predict the potential function of gut microbiota through PICRUSt analysis, however, the metagenome shotgun sequencing can identify the gut microbiota species at all taxonomic levels and reveal their functional pathways (Wang et al., 2021a). Therefore, metagenomic shotgun sequencing can be selected to further analyze the changes of gut microbiota composition and function in LPS-induced liver diseases of rats after AMPs intervention, and confirm the functional results predicted by PICRUSt.

In summary, the gut microbiota disordered have been demonstrated in the fecal microbiota of rats with liver diseases induced by LPS, which are characterized by the increase of potential pathogenic bacteria, the decrease of potential beneficial bacteria and the change of metabolic function pathways. The change of gut microbiota may be a biological factor in liver diseases and its progression. This study showed that the Aronia melanocarpa polyphenols (AMPs) could play a series of roles by modulating gut microbiota. Therefore, further researches such as gut microbiota shotgun metagenomic analysis on modulating the gut microbiota based on *Aronia melanocarpa* polyphenols (AMPs) may provide new insights into the pathogenesis of liver diseases, the etiology of its progression and new treatment strategies. We believe that the change and modulate of gut microbiota in liver diseases could help us to rich our acknowledge of liver diseases and may become the biomarkers to diagnosis and treatment liver diseases.

5 Conclusion

In conclusion, combined with our previous studies, this study showed that *A. melanocarpa* polyphenols, could alleviate the imbalance of gut microbiota and by mediating the gut-liver axis to influence the gut microbiota in rats with LPS-induced liver diseases, and providing a theoretical basis for further revealing the mechanism of gut microbiota on the occurrence and development of liver diseases and providing a new target for the treatment of liver diseases.

Conflict of interest

The authors declare that there are no conflicts of interest.

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