A Comparison of the Gut Microbiota Modulatory Effect of Pu-Erh and Dian Hong Black Tea

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Abstract

This study used Illumina Miseq high-throughput sequencing technology based on 16S rRNA V4 to explore and compare the regulatory effect of Dian Hong black tea and Pu-erh aqueous extract on the structure and diversity of the intestinal flora of mice. The results showed that the microbial communities from mice treated with water (W), Pu-erh (P), and Dian Hong black tea (R) clustered together and separated along the principal coordinate axis. In addition, the relative abundance of the two predominant microbial divisions (phylum level) of *Firmicutes* and *Bacteroidetes* in mice differed from those treated with water, Pu-erh, and Dian Hong black tea. The ratio of *Firmicutes* to *Bacteroidetes* was 2.37 in the W group and significantly increased in the P and R groups (2.63 and 3.99). According to the Venn diagrams, a total of 620 OTUs were obtained via OTU clustering, consisting of three groups. The LEfSe results indicated that 22 genera were differentially represented among the three groups, with 12 being more abundant in mice from the P group and six being more abundant in mice from the R group. In conclusion, both Dian Hong black tea and Pu-erh tea significantly regulated the intestinal flora of mice. However, no apparent differences were evident between the regulatory intestinal flora mechanisms of the two. It also suggested that there were indeed differences in the degree of oxidation and specific chemical compositions of active substances, such as polyphenols.

Introduction

Tea is one of the world's three natural beverages due to its unique color, aroma, and taste (Gaeini et al. 2019). The manufacturing methods and the degree of oxidation of tea polyphenols can be divided into six categories: green tea, white tea, yellow tea, oolong tea, black tea, and dark tea (Li et al. 2019). Tea has significant nutritional and medicinal value, and drinking tea is beneficial to human health and longevity (Hayakawa et al. 2018; Kochman et al. 2020). As such, it displays broad development prospects and contains tea protein, tea polyphenols, tea polysaccharides, caffeine, and other functional components. Tea can lower blood sugar, blood fat, blood pressure and exhibit anti-thrombosis, anti-oxidation, anti-bacterial, and anti-radiation properties while enhancing immunity (Murray et al. 2015; Tang et al. 2019). Recent research revealed that tea also influences the intestinal microbiota in addition to direct immune regulation (Sheng et al. 2018; Zhang et al. 2020).

Intestinal microbiota represents a substantial ecosystem. An average adult has 10^{13} cells of its own, while the number of bacteria in the gut is about ten times greater than the total number of somatic and germ cells (Bäckhed et al. 2005). In addition, the microbial gut genome encodes 100-fold more unique genes than the human genome (Qin et al. 2010). In recent years, increasing research involving gut microbiota revealed that intestinal microflora exchanges information and makes contact with host cells, regulating their metabolism, gene, and protein expression, antagonizing and cooperating to maintain the balance of health and disease (Lynch and Pedersen 2016; Chen et al. 2020). The imbalance or disorder of human intestinal microflora can lead to various diseases, such as diabetes, hypertension, cardiovascular and cerebrovascular diseases, metabolic syndrome, gastrointestinal inflammation, depression, and even autoimmune diseases (Angelakis et al. 2013; Backhed et al. 2004; Gill et al. 2006).

Dian Hong and Pu-erh tea are mainly produced and exported in Yunnan Province and use unique large-leaf tea leaves from the area as raw materials. Ripe Pu-erh tea is made from sun-dried crude green tea leaves via a particular processing procedure (Wei et al. 2020), while the oxidation of polyphenols is primarily achieved via damp heat. Black tea is produced from sun-dried green tea after withering, rolling, fermentation, and drying (Wei et al. 2020). During fermentation, the oxidation of polyphenols in fresh leaves is accomplished via the enzymatic action of polyphenol oxidase (PPO) and peroxidase (PO) (Samanta et al. 2015; Jin et al. 2020). Due to different processing methods, the content (mass fraction) of several main chemical components of ripe Pu-erh tea and black tea varies extensively, while their health effects on the human body are also different.

This research utilized the Illumina Miseq high-throughput sequencing technology of the 16S rRNA V4 region to compare the effect of ripe Pu-erh tea and aqueous Dian Hong black tea extract, produced from the same raw materials but via different processes, on the structure and diversity of the cecal intestinal microflora in mice. Furthermore, this study aims to explore their health care benefits of Pu-erh tea and Dian Hong black tea, providing a theoretical basis for further developing and utilizing these beverages.

Materials And Methods

Sample preparation

Here, 3 g of Pu-erh tea and Dian Hong black tea were boiled separately in 150 mL of distilled water for 30 min on an electric stove, obtaining the aqueous extract after filtering and centrifugation (Cao et al. 2013). The specific steps were as follows: First, 100–200 mL of distilled water was preheated at 99°C. Then, 3 g of tea leaves and 50 mL of distilled water were added to a 250 mL conical flask and heated on an electric stove until boiling, at which point the timing was started. Then, 50 ml of the preheated distilled water was added at 10 min and 20 min while the heating process continued. After 30 min, heating was ceased, and the mixture was filtered through gauze to obtain an aqueous extract. A constant volume of 150 mL was cooled to room temperature and transferred to a tube for centrifugation at 10000 r/min for 5 min. The supernatant was removed and stored in a refrigerator at -20°C until use.

Animal group experiment

Here, 24 mice (all mice are provided by the experimental center, 12 males and 12 females) were fed in a laboratory at a constant temperature and humidity. The conditions were controlled at a 12 hr light/dark cycle at 20–27°C and 40%-70% humidity (Lu et al. 2019). The mice were given basic meals and enough drinking water, and the cages were cleaned regularly. After 10 days of adaptive feeding, the mice were randomly divided into three groups: the blank control group (the water feeding group), the Pu-erh tea feeding group, and the Dian Hong black tea feeding group. All mice were gavaged every morning in addition to the standard diet. The mice in the Pu-erh tea feeding group and Dian Hong black tea feeding group were gavaged with 0.15 ml of Pu-erh tea and Dian Hong black tea aqueous extract, respectively, while the blank control group was given the same amount of sterilized distilled water for 15 consecutive days. During the entire experimental period, the basic condition of the mice was observed daily, and their

body weight was recorded at regular intervals of 5 days. After 15 days, the mice were sacrificed, and the cecal contents were dissected and stored at -80°C.

DNA extraction and sequencing

The internal cecal content samples (70–80 mg) were carefully acquired using sterile tweezers to avoid environmental contamination. The CTAB/SDS method was used to extract total genomic DNA. NanoDrop ND-1000 (NanoDrop Technologies) spectrophotometer was used to determine DNA concentration, and 1% agarose gel was used to detect DNA purity. PCR amplification was performed using the 515f/806r primer set (515f: 5'-GTG CCAGCMGCCGCGGTA A-3', 806r: 5'-XXX XXXGGACTACHV GGGTWT CTA AT-3') with a 6-bp error-correcting barcode unique to each sample. The PCR reactions were conducted using Phusion® High Fidelity PCR Master Mix (New England Biolabs). The purified amplified products were sent to Novartis Gene Bio-Information Technology Co., Ltd. (Beijing, China) to sequence the V4 hypervariable region of the 16S rRNA gene.

Bioinformatics and statistical analyses

The primer sequences were separated from the single-end reads, and then use the parameters recommended by Cutadapt (V1.9.1) quality-controlled process to perform quality filtering (Martin 2011). The chimera sequences (Haas et al. 2011) were removed by using the SILVA reference database (Quast et al. 2013) and the UCHIME algorithm (Edgar et al. 2011). Then use Uparse software to analyze the obtained clean data (Uparse v7.0.1001). The sequences with a similarity greater than 97% are classified as the same OTUs. The classification and assignment of each OTU representative sequence was implemented based on SILVA database and Mothur algorithm (Edgar 2013). A multiple sequence alignment analysis was conducted using MUSCLE Version 3.8.31 (Edgar 2004).

The alpha diversity analysis uses the Shannon and Simpson indexes. The unweighted/weighted UniFrac distances and Bray-Curtis distances were calculated for the Jackknifed beta diversity analysis. Principal Coordinate Analysis (PCoA) and Non-Metric Multi-Dimensional Scaling (NMDS) were constructed based on these distances (Lozupone and Knight 2005). The alpha diversity values were also compared using Wilcoxon Rank Sum Test. Bray-Curtis distance-based similarity analysis was used for the significance test of beta diversity difference between groups. The linear discriminant analysis effect size (LEfSe) was employed to determine microbial taxa featured in different groups (Segata et al. 2011). The functional profiles from the metagenomic 16S rRNA data were predicted using Tax4Fun. The student's t-test was used to identify pathways with substantial differences between the groups.

Results

Sequencing metadata

After getting rid of low-quality reads and chimeras, 1,923,284 high-quality 16S rRNA gene sequences (V4-533-786 bp) without chimera were obtained, with an average of 80136.83 ± 86.63 sequences per sample,

ranging from 80034 to 80344. The average length of these sequences is 411 bp, and they belong to 1703 operational taxonomic units (OTUs) on the basis of 97% similarity. Each sample had an average of 567.67 ± 7.09 OTUs. After calculation, the effective data accounted for 98%, while the average Q20 was 80.67, and the average GC ratio was 52.79% (Table 1).

Microbial diversity in the gastrointestinal tracts of the mice

The microbial community richness (alpha diversity) was measured using the Shannon index, Chao1, and observed species. No significant differences were observed between the P and R groups. According to the Chao1 and observed species analysis, significant differences were found in the bacterial community structure of P vs. W and R vs. W (Fig. 1, $P<0.05$).

To examine beta diversity among mice in W, P, and R groups, weighted and unweighted UniFrac distances were both calculated to estimate dissimilarities in the community membership. PCoA was applied to visualize distances, showing that mice treated with water, Pu-erh, and Dian Hong black tea harbored distinct microbial taxa (Wang et al. 2020) (Fig. 2). Based on membership, the microbial communities of mice treated with water, Pu-erh, and Dian Hong black tea, clustered together and were separated from each other along the principal coordinate axis. Differences in community memberships between the different groups were also proven to be significant by ANOSIM ($P < 0.01$), indicating distinct microbial community structures among mice treated with different teas.

Differences in the microbial communities of mice treated with water, black tea, and Pu-erh

The specific taxonomic groups of species (e.g., kingdom, phylum, class, order, family, genus, and species) were classified (Fig. 3). Two groups of bacteria dominated the intestinal microbiota of different groups of mice, namely *Firmicutes* and *Bacteroidetes*, which accounted for more than 97% of reads. The relative abundance of these two predominant microbial divisions (phylum level) differed between the mice treated with water, Pu-erh, and Dian Hong black tea. The mice in the W, P, and R groups contained 67.48%, 70.11%, and 77.65% *Firmicutes*, and 28.41%, 26.65%, and 19.44% *Bacteroidetes*. At the genus level, *Lactobacillus*, *Lachnospiraceae*, *Ruminococcaceae*, *Alistipes*, *Alloprevotella*, *Turicibacter*, *Bacteroides*, *Desulfovibrio*, *Faecalibaculum*, and *Parasutterella* were dominant.

According to the Venn diagrams, a total of 620 OTUs are obtained via OTU clustering, consisting of three groups. The cluster numbers of OTUs in the Dian Hong black tea group, the control group, and the Pu-erh tea group were 569, 560, and 574. Of these, the Dian Hong black tea group and the control group shared 528 OTUs in the overlapping portion, the Pu-erh tea group and the control group shared 531 OTUs, and the Pu-erh tea group and the Dian Hong black tea group shared 532 OTUs. Moreover, 19 species were unique to the Pu-erh tea group, nine species were unique to the control group, and 17 species were unique to the Dian Hong black tea group (Fig. 4).

LEfSe was employed to identify specific genera that were differentially distributed among the mice in the W, P, and R groups, presenting a potent tool that focuses on the significance of differences, as well as

biological relevance. The LEfSe results are shown in Fig. 5. Furthermore, 22 genera were differentially represented among the three groups, with 12 being more abundant in the mice of the P group (e.g., *Lactobacillaceae*, *Lactobacillus spp.*, *Bacilli*, *Lactobacillales*, *Ruminococcaceae*, *Clostridium papyrosolvens*, *Ruminococcaceae spp.*, *Erysipelotrichales*, *Erysipelotrichia*, *Alloprevotella spp.* and *Prevotellaceae*) and six being more abundant in the mice of the R group (e.g., *Lachnospiraceae*, *Lachnospiraceae spp.*, *Clostridiales*, *Clostridia*, *Firmicutes*, and *Clostridiales bacterium CIEAF 020*).

Discussion

Dian Hong and Pu-erh are both famous tea originating in Yunnan, China. Due to their long history, unique production process, and outstanding health benefits, both tea have been selected as representative of the intangible national and cultural heritage of the country. Pu-erh as a dark tea is a fully fermented tea, and the development of its quality characteristics depends on the types of compounds contained in the fresh, raw leaf material. The most significant impact on the flavor of black tea comes from tea polyphenols, especially catechins and flavonols. Polyphenols, especially catechins, undergo a strong oxidation reaction when exposed to PPO to generate theaflavins, thearubicins, and brown pigments (Gong et al. 2012). The unique color, aroma, taste, and other quality characteristics of black tea develop via a series of coupling oxidation reactions. Dian Hong and Pu-erh are made with fresh leaves from large-leaf tea plants in Yunnan as raw materials. Yunnan big-leaf tea leaves contain high levels of tea polyphenols, of which catechins account for 70% of the total amount, while the general small-leaf tea trees exhibit a relatively low content of fresh-leaf tea polyphenols. Generally, of the catechin compounds in tea, the L-EGCG content is the highest (Almatroodi et al. 2020), followed by L-ECG and L-EGC. However, the content of L-ECG in Yunnan large-leaf species is almost close to L-EGCG, and the content of L-EC is similar to that of L-EGC and even exceeds the content of L-EGC. At the same time, the ratio of ester catechins of catechins of large-leaf species is greater than that of catechins of small-leaf species, which makes large-leaf tea leaves superior in vitro oxidation resistance than small-leaf species. However, some differences are evident between the fermentation processes of the two teas, leading to specific variations in the nutritional composition and flavor of the finished tea. Pu-erh tea is produced via a unique fermentation process (post-microbial fermentation). The post-fermentation process involves microorganisms, allowing the chemical components of tea raw materials to undergo more complex biotransformation in a high-temperature and high-humidity environment, to form theabrownins (Gong et al. 2012) and puerin(8-C N-ethyl-2-pyrrolidinone substituted flavan-3-ols) (Wang et al. 2014), statins (Yang and Hwang 2006; Jeng et al. 2007), gallic acid (Zhang et al. 2020), flavonoids (Beresniak et al. 2012; Li et al. 2021; Zhu et al. 2020) and other chemical quality components and biologically active compounds.

Currently, extensive research focuses on the health effects of dark tea and its main chemical components. Beresniak et al. (2012) investigated the relationship between black tea consumption and significant health indicators in 50 countries worldwide. The results showed that drinking black tea is negatively correlated with the course of diabetes. The linear correlation model showed a significant correlation between regular black tea consumption and low diabetes prevalence. Ramadan et al. (2009) studied the regulating effect of black tea water extract on hyperglycemia, indicating that black tea

inhibited this condition. However, the specific chemical composition, target, and mechanism remain unclear. The theaflavins and thearubigins in black tea can scavenge free radicals and act as antioxidants. Wang et al. (2007) found that five different polar thearubigins could scavenge DPPH free radicals, showing a certain dose-effect relationship. At a concentration of 66.7μg/ml, the partial clearance rate of the five different thearubigin polarities exceeded 85%. Ma et al. (2009) revealed that theaflavins could improve myocardial infarction size in rats after ischemia/reperfusion injury in a dose-dependent manner. Theaflavins can increase the SOD activity of the myocardium after ischemia/reperfusion in rats while reducing MDA content. Studies have shown that theaflavins distinctly affect anti-myocardial ischemia/reperfusion injury, which is related to their antioxidative effect. Li et al. (2009) found that theaflavins can significantly inhibit the phosphorylation activity of p38 mitogen-activated protein kinase (p38MAPK) and the expression of transforming growth factor-β protein, reducing accumulation in the extracellular matrix. It shows that theaflavins can reduce the synthesis of the extracellular matrix by regulating the p38MAPK signal transduction pathway, delaying diabetic glomerular hypertrophy and glomerular sclerosis. As a type of dark tea, Pu-erh tea not only has the health characteristics of most dark teas, but recent studies have shown that Pu-erh tea is particularly effective in reducing weight, fat, and blood sugar. Studies have shown that Pu-erh tea can reduce LDL-C levels in the blood of SD rats while increasing the HDL-C level. Green tea, black tea, and oolong tea have been shown to only reduce these levels at the same time (Kuo et al. 2005). Pu-erh tea also regulated has the absorption of long-chain fatty acids and tight junction proteins in the intestines of NAFLD rats, improving liver steatosis (Zhu et al. 2016).

The health effects of black tea not only include direct regulation of human health, but also very important in the regulation of intestinal flora. A large number of different microorganisms live in the large intestine, mainly anaerobic bacteria. Generally, there are more than 1,000 "species-level" phylotypes, belonging to a few phyla, that exist in a healthy intestine (Lozupone et al. 2012). Among these phyla, the dominant microorganisms are usually *Bacteroidetes* and *Firmicutes*, followed by *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* (Lozupone et al. 2012). They play a crucial part in maintaining the balance of intestinal flora and human health. They absorb nutrients and energy through diet, thereby improving the body's immune function. Those unabsorbed phenols will be degraded by intestinal microbial enzymes after arriving the colon, generating series metabolites that are easily absorbed, and may also

regulate the composition of the intestinal microbiota (Liu et al. 2018). The interaction between these phenolics and the gut microbiota may bring some health benefits to the human body.

Conclusion

This study focused on the impact of Dian Hong and Pu-erh tea on the intestinal micro-ecological balance. Comparing and analyzing the microbial changes in mice after gavage with two kinds of black tea revealed their influence on the intestinal flora. This provided an experimental basis for further elucidating the relationship between the two kinds of tea and human health. The results show that Dian Hong and Pu-erh tea have a substantial regulatory effect on the cecal flora of mice while also displaying significant

regulatory differences. Weighted and unweighted UniFrac distances showed that mice treated with water, Pu-erh, and Dian Hong black tea exhibited distinct microbial taxa. The microbial communities of the mice in the W, P, and R groups clustered together and were separated from each other along the principal coordinate axis. Furthermore, microbial community richness (alpha diversity) showed that the bacterial community structures in the P and R groups were significantly increased compared with the W group. The ratio of *Firmicutes* to *Bacteroidetes* was 2.37 in the W group and was significantly higher in the P and R groups (2.63 and 3.99). The consumption of black tea substantially increases the level of Lactobacillus in the colonic flora, which is critical to intestinal health. This may be related to the ability of black tea to lower blood lipids and blood sugar and prevent colon cancer. The effect of tea phenolics on certain intestinal bacteria might be connected with the ability of these microorganisms to metabolize flavonoids. For instance, certain *Lactobacilli* can use phenolic compounds to acquire energy, and the selective growth stimulating effect of this phenolic substance on *Lactobacillus* is similar to that of substances such as inulin and galactooligosaccharides, which are commonly called prebiotics. In conclusion, both Dian Hong and Pu-erh tea can significantly regulate the intestinal flora of mice. However, there are obvious differences between the two regarding the regulatory mechanism of the intestinal flora, implying that these two teas vary in compounds, such as polyphenols, due to different fermentation levels. There are certain differences in the degree of oxidation and specific chemical composition of the substances. In order to better understand the positive effects of these two teas on human health, it is necessary to conduct a more extensive and in-depth study of the interaction between tea phenols and gut microbes.

Declarations

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Author Contributions

The study was designed by YT, HZ and NW; BZ, ZPY and HLW carried out the research; BZ, CHG and KLX analyzed the data; YHW and CHG collected information; YT, NW and BZ prepared the manuscript.

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Availability of data and materials

All data about this research are in this manuscript.

Ethics approval and consent to participate

The experimental procedures of this study have been reviewed and approved by the Institute of Animal Health Animal Care and Use Committee of Xihua University (Chengdu, China, approval number: 2019-018, 11 March 2019). And animals were raised strictly in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (approved by the State Council of the People's Republic of China).

Consent for publication

All authors have approved this manuscript, and confirmed that this research has not been published before, nor is it considered for publication elsewhere.

Competing interests

The authors declare there are no conflicts of interest.

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Tables

Table 1 Sequencing information

#Sample Name	Raw_Reads (#)	Clean_Reads (#)	Base (nt)	AvgLen (nt)	Q20	GC%	Effective%
W1	80138	80138	32967865	411	78.26	52.93	100
W2	80115	80115	32835944	409	77.7	52.7	100
W3	80117	80117	33147169	413	78.89	52.9	100
W4	80065	80065	32881418	410	77.48	52.69	100
W5	80124	80124	33028899	412	76.48	52.57	100
W6	80213	80213	32910713	410	75.72	53.36	100
W7	80063	80063	32753434	409	77.72	52.94	100
W8	80176	80176	32923558	410	76.68	53.11	100
P1	88066	80284	32866220	409	84.54	52.52	91.16
P2	87578	80289	33209376	413	84.9	52.38	91.68
P3	82649	80042	32917100	411	85.23	52.32	96.85
P4	83681	80232	32914567	410	84.88	52.35	95.88
P5	83190	80075	32720333	408	84.74	52.41	96.26
P6	86588	80134	32931179	410	83.35	52.13	92.55
P7	88927	80060	33007306	412	85.27	52.18	90.03
P8	86597	80344	33184453	413	85.54	52.05	92.78
R1	80237	80237	33097154	412	79.13	52.94	100
R2	80092	80092	33111533	413	80.99	53.09	100
R3	80052	80052	32838845	410	79.66	53.1	100
R4	80096	80096	32887856	410	79.59	53.34	100
R5	80034	80034	33093350	413	80.52	52.82	100
R6	80140	80140	32999059	411	79.33	53.31	100
R7	80095	80095	32890101	410	79.66	53.41	100
R8	80067	80067	32901858	410	79.82	53.33	100

Figures

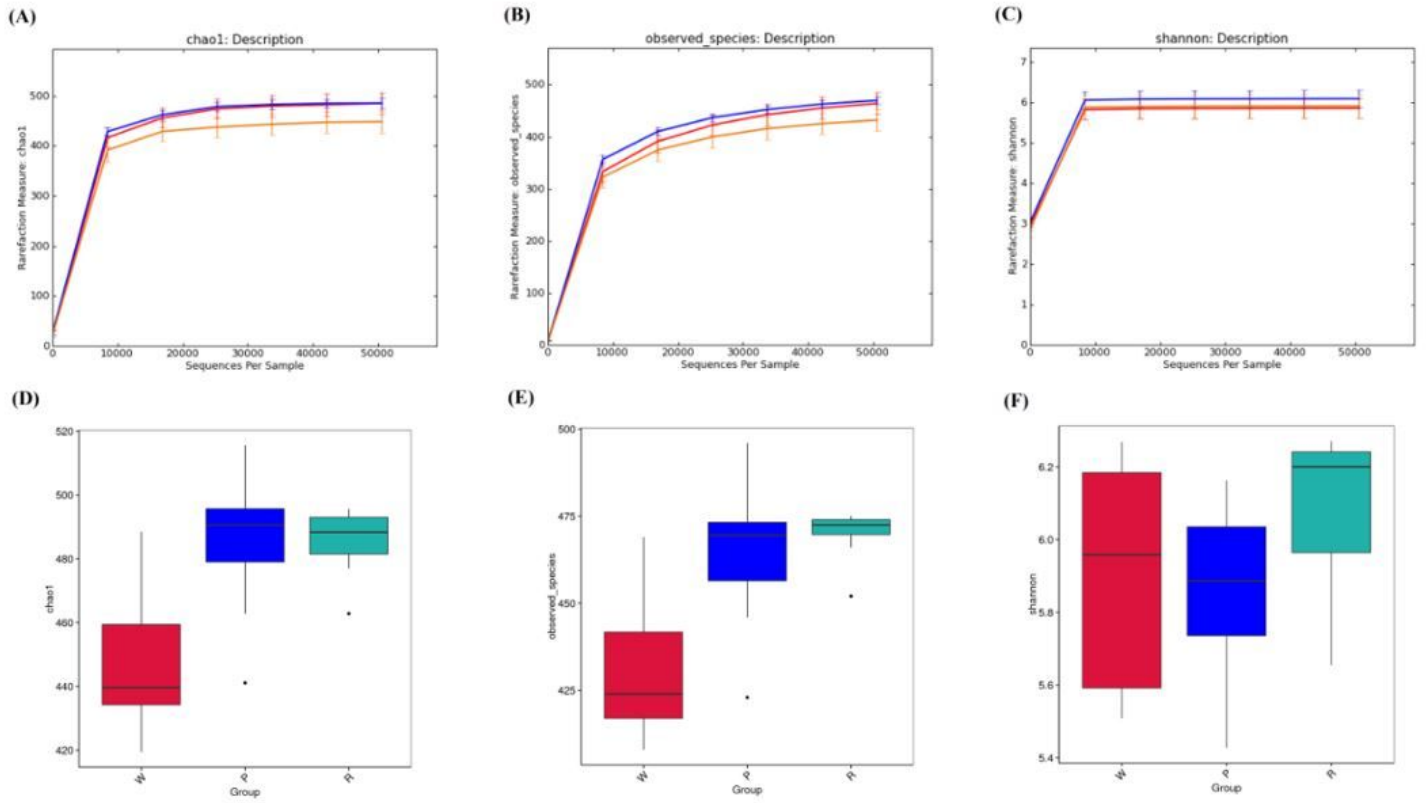

Figure 1

Microbial community richness and the bacterialcommunity structure of mice in the W, P, and R groups.

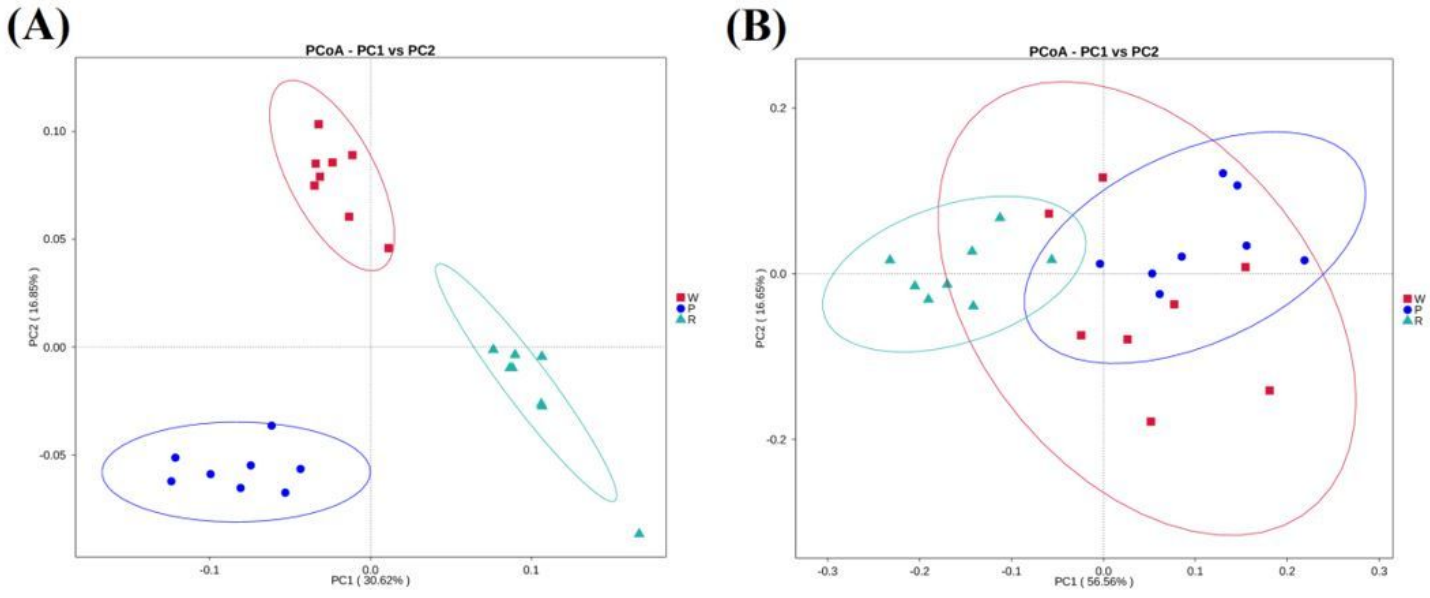

Figure 2

Differences in the microbial communities of mice in the W, P, and R groups.

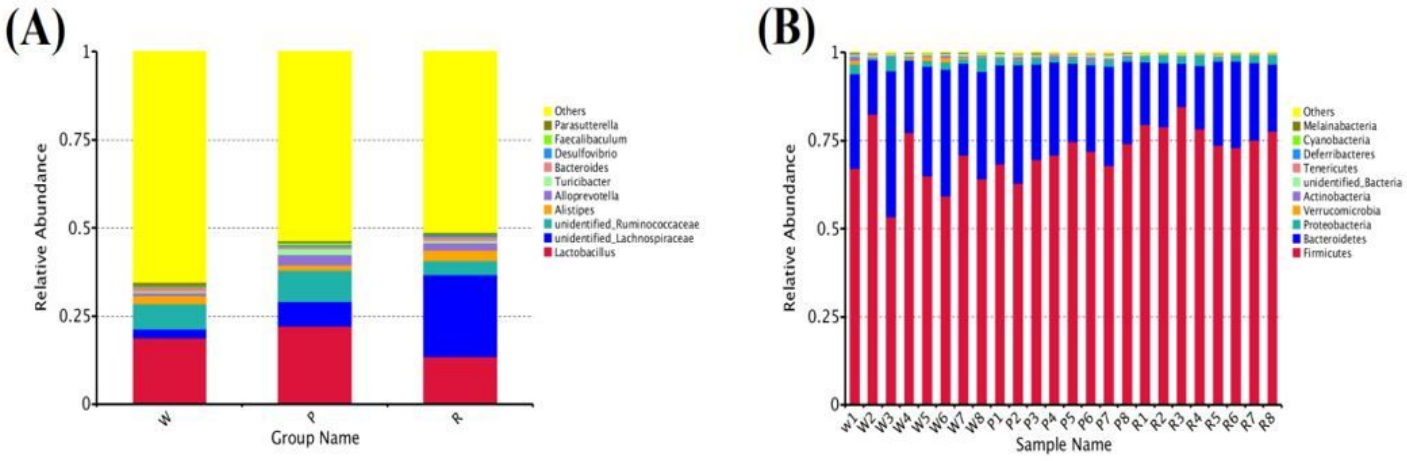

Figure 3

The classification of specific taxonomic groups of species of the mice in the W, P, and R groups.

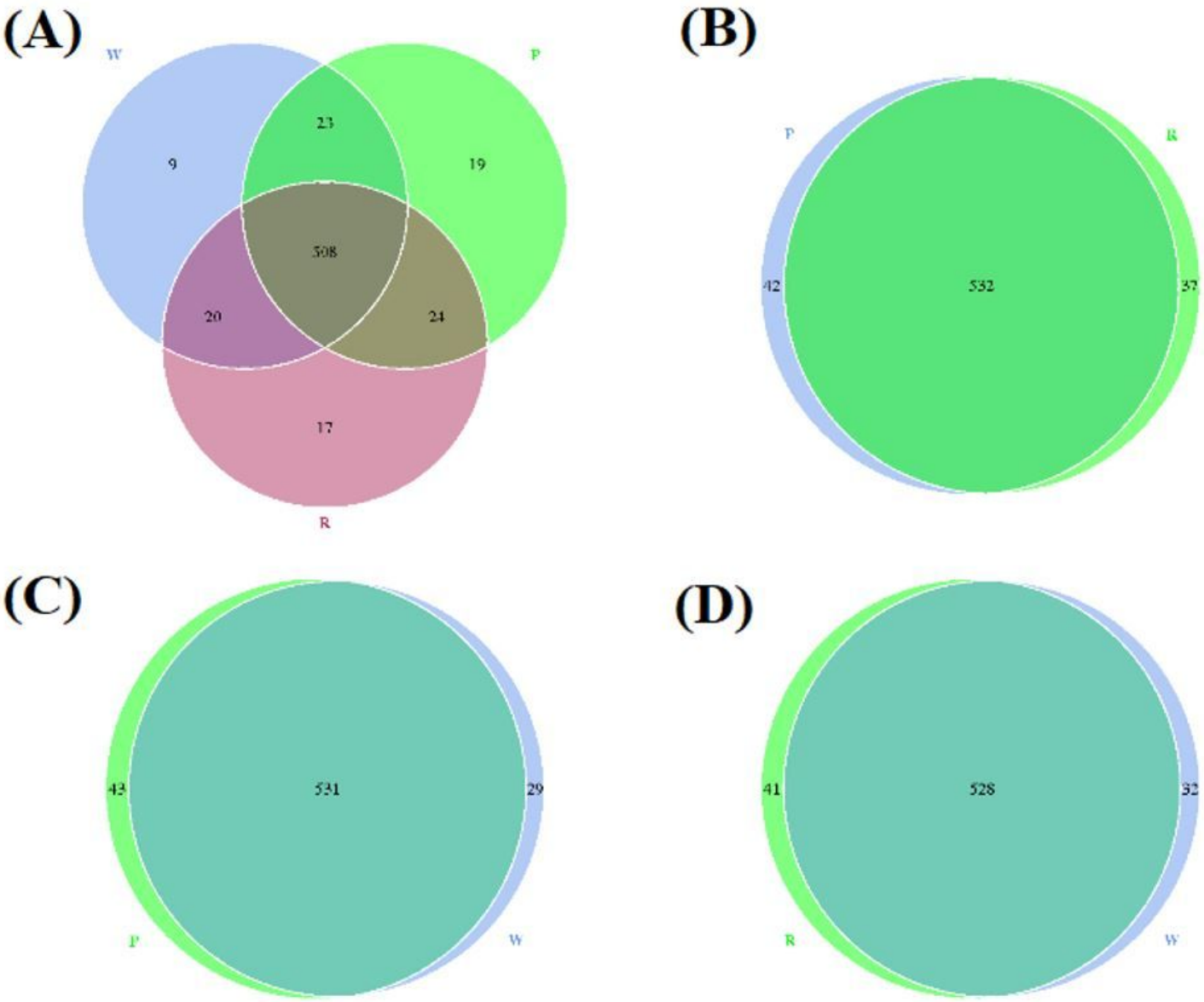

Figure 4

Venn diagrams of mice in the W, P, and R groups.

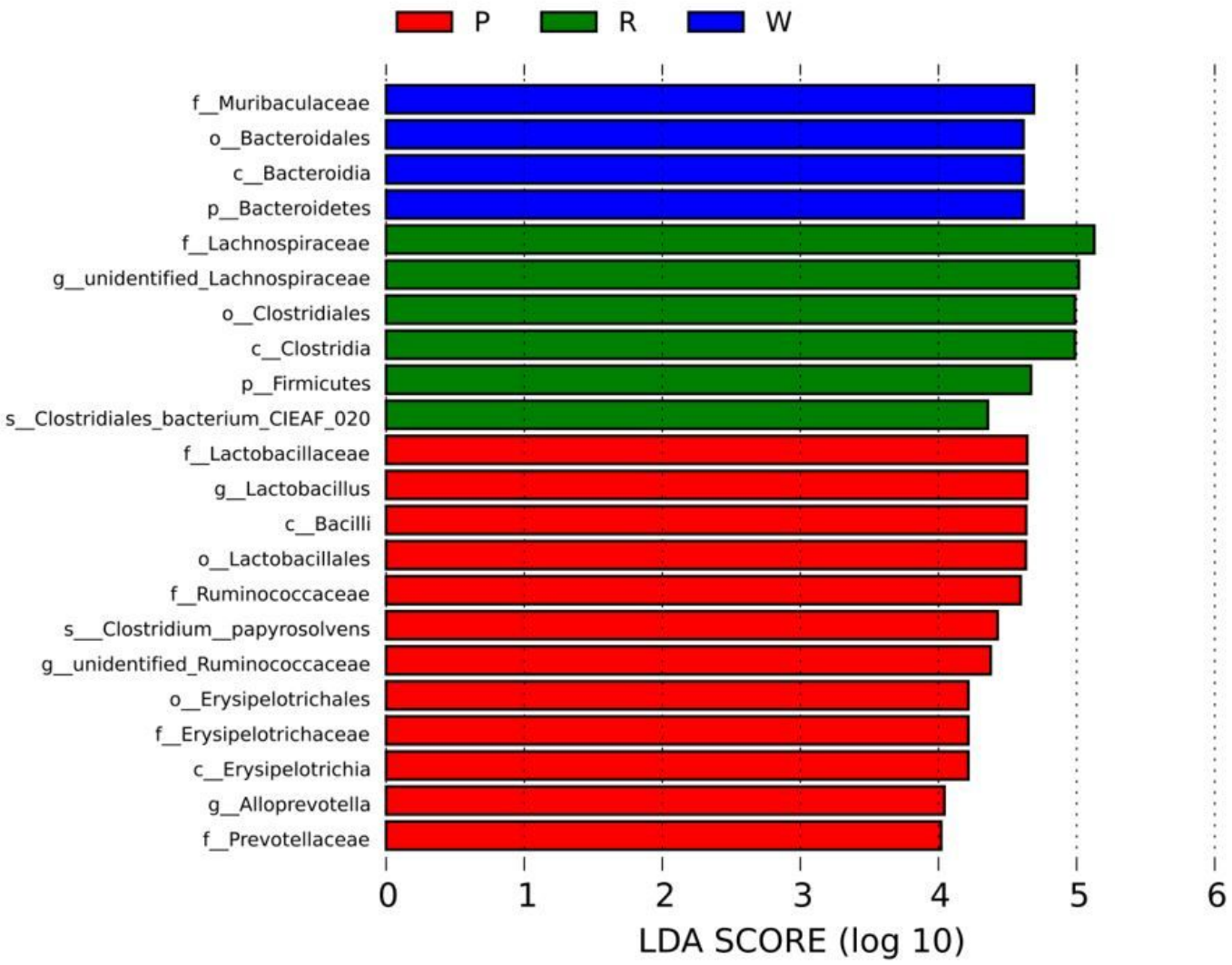

Figure 5

LEfSe of the mice in the W, P, and R groups.