



# Effects of Different Treatment Methods of Dried Citrus Peel (*Chenpi*) on Intestinal Microflora and Short-Chain Fatty Acids in Healthy Mice

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*Chenpi* is a kind of dried citrus peel from *Citrus reticulata*, and it is often used as traditional Chinese medicine to treat dyspepsia and respiratory tract inflammation. In this study, to determine which way of *chenpi* treatment plays a better effect on the prevention of obesity in healthy mice, we conducted 16S ribosomal RNA (rRNA) gene sequencing for intestinal microbiota and gas chromatography-mass spectrometry detector (GC/MSD) analysis for short-chain fatty acids (SCFAs) of female rats fed with either *chenpi* decoction or *chenpi* powder-based diet ( $n = 10$  per group) for 3 weeks. *Chenpi* powder (CP) group significantly reduced abdominal adipose tissues, subcutaneous adipose tissue, and the serum level of total triacylglycerol (TG). At a deeper level, *chenpi* powder has a better tendency to increase the ratio of *Bacteroidetes* to *Firmicutes*. It alters the *Muribaculaceae* and *Muribaculum* in intestinal microbiota, though it is not significant. The concentrations of acetic acid, valeric acid, and butyric acid increased slightly but not significantly in the CP group. *Chenpi* decoction just reduced perirenal adipose tissues, but it shows better antioxidant activity. It has little effect on intestinal microbiota. No differences were found for SCFAs in the *chenpi* decoction (CD) group. The results indicated that *chenpi* powder has a better effect in preventing obesity in mice. It can provide a basis for the development of functional products related to *chenpi* powder.

**Keywords:** *chenpi* powder, *chenpi* decoction, intestinal microbiota, short chain fatty acids, different treatment methods

## INTRODUCTION

Dried citrus peel (*chenpi*) is the mature dry pericarp of *Citrus reticulata*. As a traditional Chinese medicine, it has a good effect on treating dyspepsia and improving respiratory tract inflammation. *Chenpi* contains many active components, such as essential oil (1), flavonoid (2), pectin (3), insoluble fiber (4), and so on. Citrus peel essential oils may ameliorate hypercholesterolemia and hepatic steatosis by modulating lipid and cholesterol homeostasis, and most of them have good

antimicrobial and antioxidant activities (5, 6). Polymethoxyflavones, a kind of flavonoid from citrus peel, have anti-obesity, anti-hyperglycemic, and antiviral activities; meanwhile, it may effectively prevent the progression of metabolic syndrome (7–10). Pectin polysaccharide has *in vitro* intestinal immunomodulatory activity (11). In addition to the abovementioned active substances, pure *chenpi* powder also contains a large amount of dietary fiber. The composition and activity of intestinal microbiota and the production of short-chain fatty acids (SCFAs) were affected by dietary fiber (12). Meanwhile, the production of SCFAs (in particular, acetate, propionate, and butyrate) is closely related to intestinal health and function (13).

Intestinal microbiota are microorganisms colonized in the human digestive tract, which is closely related to age, obesity, and inflammation (14–16). In recent years, the study on intestinal microbiota is a hot spot. Diet has different effects on intestinal microflora. More and more evidence shows that intestinal microflora is closely related to metabolism, host gene expression, and other factors (17–19). *Chenpi* has been proven to have a modulation effect on the composition of intestinal microbiota species, the abundance of microbiota, fecal SCFAs, intestinal barrier function, and gastrointestinal inflammation (20–22).

Obesity as a thorny issue worldwide is caused by many factors. Obesity can cause a series of complications, such as hypertension, hyperlipidemia, metabolic diseases, and increasing organ burden (23, 24). Several studies have observed the effects of extracts or natural products on intestinal microorganisms, SCFAs, glucose metabolism, and body weight of healthy mice model (25, 26). Looking for natural products that can alleviate and treat obesity is a healthy and safe method. Although there are some studies on the effect of reducing weight and lipid of *chenpi*, there is no study on which way of *chenpi* treatment can play a better effect. In this experiment, we observed the effect of the *chenpi* on healthy mice. Traditionally, *chenpi* was infused with boiling water to extract their effective components such as “decoction.” In this study, we added *chenpi* to the normal diet of mice in two forms, both *chenpi* decoction and *chenpi* powder. This study aimed to investigate the modulation effect of two different types of *chenpi* on the accumulation of adipose, intestinal microbiota, antioxidant capacity, and SCFAs to unveil their potential application for obesity prevention, which may also provide a basis for the use of *chenpi* as a kind of anti-obesity food in the food industry.

## MATERIALS AND METHODS

### Mice and Housing

Forty four-week-old C57BL/six female mice (Tianqin Biotechnology Company, Changsha, China) were housed in a controlled room with a 12 h/day lighting cycle during the experimentation. Food and drinking water were freely available to mice. Following 1 week of acclimation, mice ( $n = 10$ ) were randomly grouped to control (C), *chenpi* decoction (CD), control powder (P), *chenpi* powder (CP). They were all provided with a normal diet. The normal diet contained 54.9% corn, 5.6% casein, 18% soybean meal, 6.5% beer yeast, 0.7% lard, 0.8% bean oil, 0.5%

salt, 1.4% fishmeal, and 1% premixture. The difference between granulated (C) and powder (P) groups is whether granulation is carried out. In the CD group and CP group, *chenpi* decoction and *chenpi* powder, respectively, were added to the normal diet. The body weight, food intake, and water intake were recorded once a week. After 3 weeks of administration, blood samples were collected by orbital bleeding. Liver, abdominal adipose tissues, subcutaneous adipose tissues, and perirenal adipose tissues were weighed and collected. Fecal samples were collected by 16S ribosomal RNA (rRNA) sequencing and analysis of SCFAs. The experimental protocol was approved by the Animal Care and Use Committee of Hunan Agricultural University.

### Preparation of *Chenpi* Decoction and *Chenpi* Powder

*Chenpi* was purchased from Jiangmen Xinhui tangerine peel village market limited company, Guangdong Province. The variety of *chenpi* is red *Pericarpium Citri Reticulatae*, which is made by traditional sunlight drying. According to the traditional decocting method, 10 g *chenpi* was crushed into a coarse powder and 200 ml of water was added and boiled over 95°C for 30 min. The filtrate was filtered out and then added 20 times of water to decoct again in the same way. The filtrate was combined, evaporated, and concentrated to 10 ml and stored at 4°C. The concentration of *chenpi* decoction was 1 g/ml. CD group were administered 0.2 ml/day *chenpi* decoction by gavage. The mice in the C group were given distilled water at the same time. After grinding and sieving, the *chenpi* powder was sealed in vacuum and stored at 4°C. The CP group were given 0.2 g/day *chenpi* powder in the diet.

### Histopathological Observation

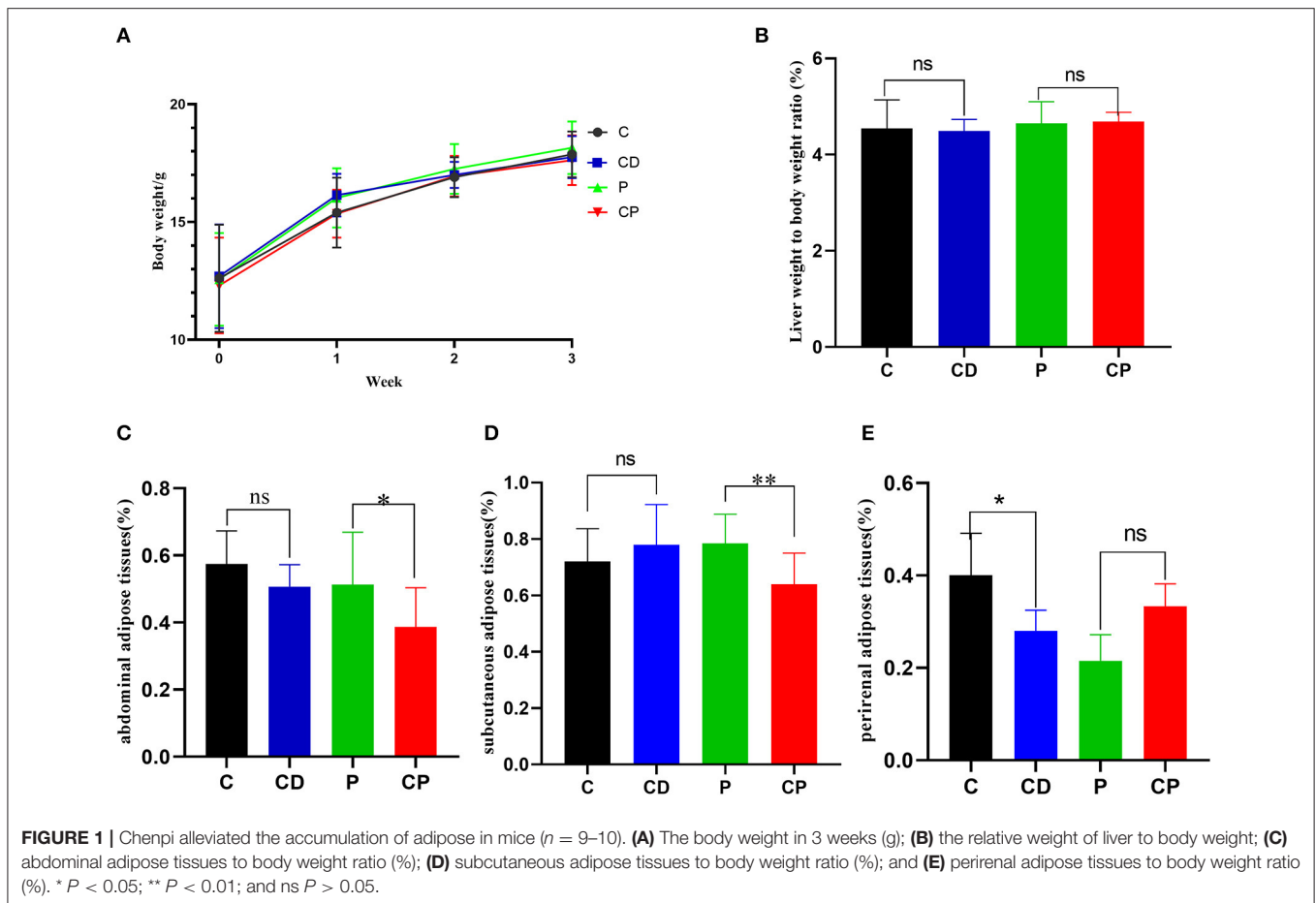
Paraformaldehyde solution in 4% was used to fix adipose tissues. Then, they were dehydrated by ethanol solution, embedded, and prepared. The subcutaneous adipose tissue was stained with H&E. Images were obtained using a Nikon Eclipse E100 Upright optical microscope from Nikon Corporation, Japan (27).

### Biochemical Analysis

The serum concentration of total cholesterol (TC), total triacylglycerol (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were determined by using Kehua biological automatic biochemical analyzer. Biochemical kits were purchased from Shanghai Kehua Bio-Engineering Co., Ltd (Shanghai, China) (28).

### Measurement of Hepatic Malondialdehyde (MDA) and Superoxide Dismutase (SOD) Levels

About 0.5 g of each liver tissue was homogenized in 4.5 ml frozen normal saline and then centrifuged and collected supernatant at 2,000 rpm for 10 min at 4°C for measurements. All these biochemical markers were measured using kits purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Coomassie Brilliant Blue was used to determine the concentration of protein (27). Each sample has a parallel sample.



## 16S Ribosomal RNA (rRNA) Gene Sequencing for Microbiota Profiling

Total genomic DNA was extracted from fecal samples and stored at  $-20^{\circ}\text{C}$  using the DNA kit according to the instructions for 16S rRNA gene pyrosequencing. Paired-end sequencing was performed on the Illumina MiSeq platform (29). The V3-V4 regions were amplified using a specific primer with the barcode by thermocycler PCR system. In summary,  $\alpha$ -diversity,  $\beta$ -diversity, and principal coordinate analysis (PCoA) were calculated and generated by Quantitative Insights Into Microbial Ecology (QIIME). The measurement was repeated three times for each sample. The online platform of Majorbio Cloud (<http://www.majorbio.com/>) was used to analyze data (30, 31).

## Detection of SCFAs

A total of 100 mg feces were dissolved in 0.9 ml water, then mixed, and then centrifuged at 13,200 g force for 10 min at  $4^{\circ}\text{C}$ . A  $1 \mu\text{l}$  supernatant of each sample was injected into the inlet for gas chromatography-mass spectrometry detector (GC/MSD) analysis. The levels of acetic, propionic, butyric, valeric, isobutyric, and isovaleric acids in SCFAs were measured using 8890B-5977B GC/MSD (Agilent Technologies Inc. CA, USA) (32, 33). The measurement was repeated three times for each sample.

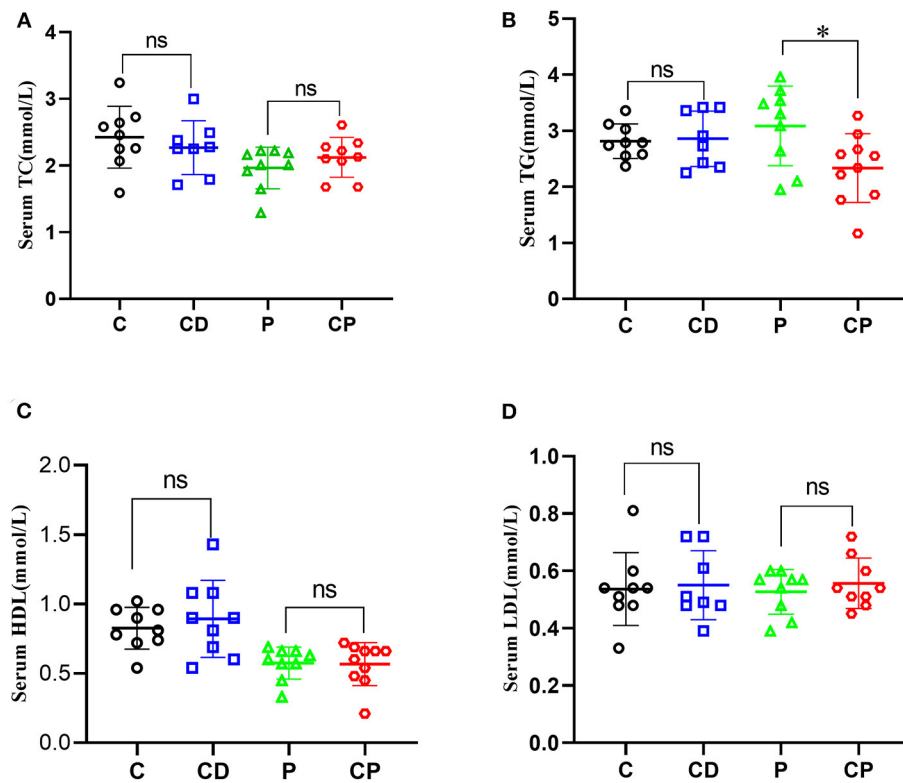
## Statistical Analysis

The statistical analyses were completed using IBM SPSS Statistics 26.0. The  $t$ -test was performed to determine the difference between groups. Values of  $P < 0.05$  mean statistically significant.

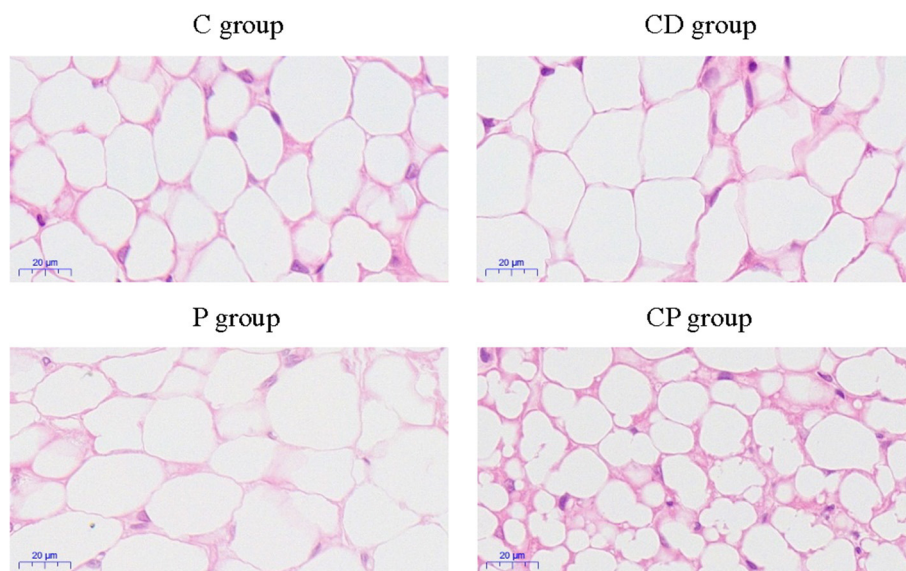
## RESULTS

### Chenpi Alleviates Accumulation of Adipose in Mice

To determine the anti-obesity effect of *chenpi* on mice, body weight, liver, abdominal adipose tissues, subcutaneous adipose tissues, and perirenal adipose tissues were weighed. *Chenpi* treatment has a tendency to regulate body weight, but the difference was not significant ( $P > 0.05$ ) (Figure 1A). Liver weight has basically no change in every group (Figure 1B). Weight of abdominal adipose tissues and subcutaneous adipose tissue was significantly reduced at 0.13 and 0.15 in the CP group compared with the P group ( $P < 0.05$ ) (Figures 1C,D). Perirenal adipose tissues were significantly reduced in CD ( $P < 0.05$ ) compared with the C group (Figure 1E). Serum concentrations of lipids were analyzed (Figure 2) to find out that *chenpi* powder can significantly reduce the serum level of TG by 24% compared to the P group ( $P < 0.05$ ) (Figure 2B) but had no



**FIGURE 2** | Chenpi adjusted serum concentrations of lipids. Concentrations of total cholesterol (TC) (A), total triacylglycerol (TG) (B), high-density lipoprotein cholesterol (HDL-C) (C), and low-density lipoprotein cholesterol (LDL-C) (D) in serum ( $n = 8-10$ ). \*  $P < 0.05$ ; ns  $P > 0.05$ .



**FIGURE 3** | The observation of subcutaneous adipose tissues by H&E staining of four treatment groups ( $\times 400$ ).

remarkable effect on the serum levels of TC, LDL-C, and HDL-C. These items showed no significant changes in the CD group compared to the C group. The histopathological observation of

adipose tissues showed that the CP group exhibited a strong inhibitory effect on the enlargement of adipocytes compared with the P group, while the difference was not significant in other

groups (Figure 3). To sum up, compared with *chenpi* decoction, supplementation of *chenpi* powder in the diet significantly alleviated accumulation of lipid and serum TG metabolism, and it reduced the relative weight of abdominal adipose tissue and subcutaneous adipose tissue.

### Chenpi Enhanced Antioxidant Capacity in the Liver

In order to test the antioxidant capacity of each group, the MDA index and SOD index of the liver were detected. The content of MDA was decreased in the CD group compared to the C group, while the content of MDA in the CP group was 1.35 nmol/mg higher than that of the P group ( $P < 0.05$ ) (Figure 4). The activity of SOD was increased marginally in the CD group compared to the C group ( $P > 0.05$ ).

### Chenpi Modulated the Structural Composition of Intestinal Microbiota

Intestinal microbiota were known as a key factor in modulating obesity. Thus, to investigate whether *chenpi* influences the intestinal microbiota of mice, 16S rRNA sequencing was tested. We analyzed the composition and difference of intestinal microflora in different diet groups.

Microbial diversity and richness were evaluated by  $\alpha$ -diversity and  $\beta$ -diversity. PCoA plot was applied to evaluate overall differences in  $\beta$ -diversity in unweighted UniFrac distance for the sample set (34, 35). As shown in Figure 5, different diets have strong effects on the gut microbial composition revealed by a clear separation among four groups. Shannon and Simpson's indexes evaluated the diversity of the microbiota. ACE and Chao indexes described the richness of the microbiota (36). As shown in Figure 6, the CD group exhibited a higher richness of microbiota evidenced by the increased ACE and Chao indexes compared to C ( $P > 0.05$ ) (Figures 6C,D) but with no significant difference. Simpson's index in the CP group significantly increased, but other indexes reduced.

As shown in Figure 7, there were differences in microbial composition among the four groups at phylum, family, and

genus levels. *Firmicutes* and *Bacteroidetes* are the two majorities at the phylum level. CD group had a 51% higher ratio of *Firmicutes* to *Bacteroidetes* compared with the C group ( $P > 0.05$ ) (Figures 7A, 8A). However, the CP group had a lower abundance of *Firmicutes* ( $P = 0.07$ ) and a higher abundance of *Bacteroidetes* ( $P = 0.06$ ) compared with the P group (Figures 7A, 8B). The difference is not significant. The dominant genera are *Muribaculaceae*, *Lactobacillaceae*, and *Lachnobacterium* at the family level. The relative abundance of *Lactobacillaceae* in the CD group is higher than in the C group ( $p > 0.05$ ) (Figures 7B, 8C). The relative abundance of *Muribaculaceae* in two powder groups is higher than in two decoction groups (Figure 7B). The relative abundance of *Muribaculaceae* increased in the CP group compared with the P group ( $P = 0.086$ ) (Figures 7B, 8D). Similar alterations were observed for *norank\_f\_Muribaculaceae*, *Lactobacillus*, and *Lachnospiraceae\_NK4A316\_group* at the genus level (Figures 7C, 8E). The relative abundance of *Muribaculaceae* ( $p = 0.09$ ) and *Muribaculum* increased in the CP group compared with the P group ( $P = 0.08$ ) (Figure 8F).

### Chenpi Changed the Content of SCFAs in Feces

The content of SCFAs of feces is closely related to intestinal health. Here, the contents of acetic, propionic, butyric, valeric, isobutyric, and isovaleric acids were tested by GC/MSD. On the whole, the content of SCFAs in the two powder groups was higher than that in the decoction groups. There was no difference in the concentration of any SCFAs in feces in the CD group when compared with the control group. The group that consumed *chenpi* powder had higher concentrations of SCFAs than the P group, especially acetic, valeric, and butyric acids, but the difference was not significant (Figure 9).

## DISCUSSION

We present the results of a study investigating the effects of different supplementation treatments with *chenpi* on various

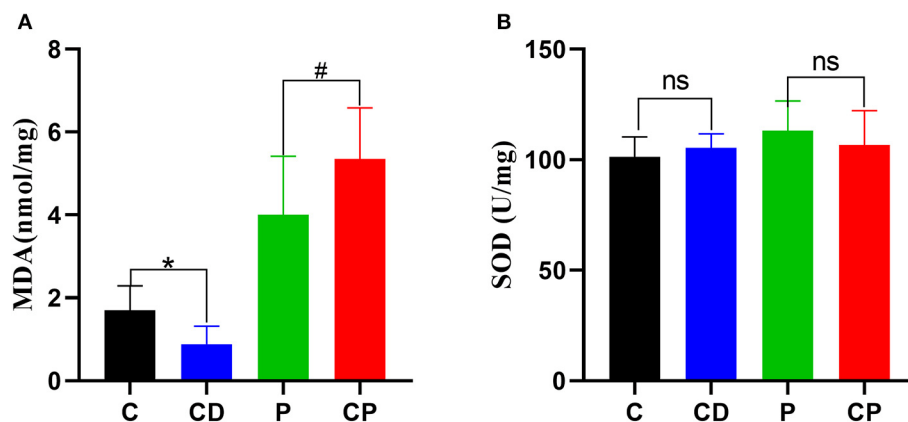
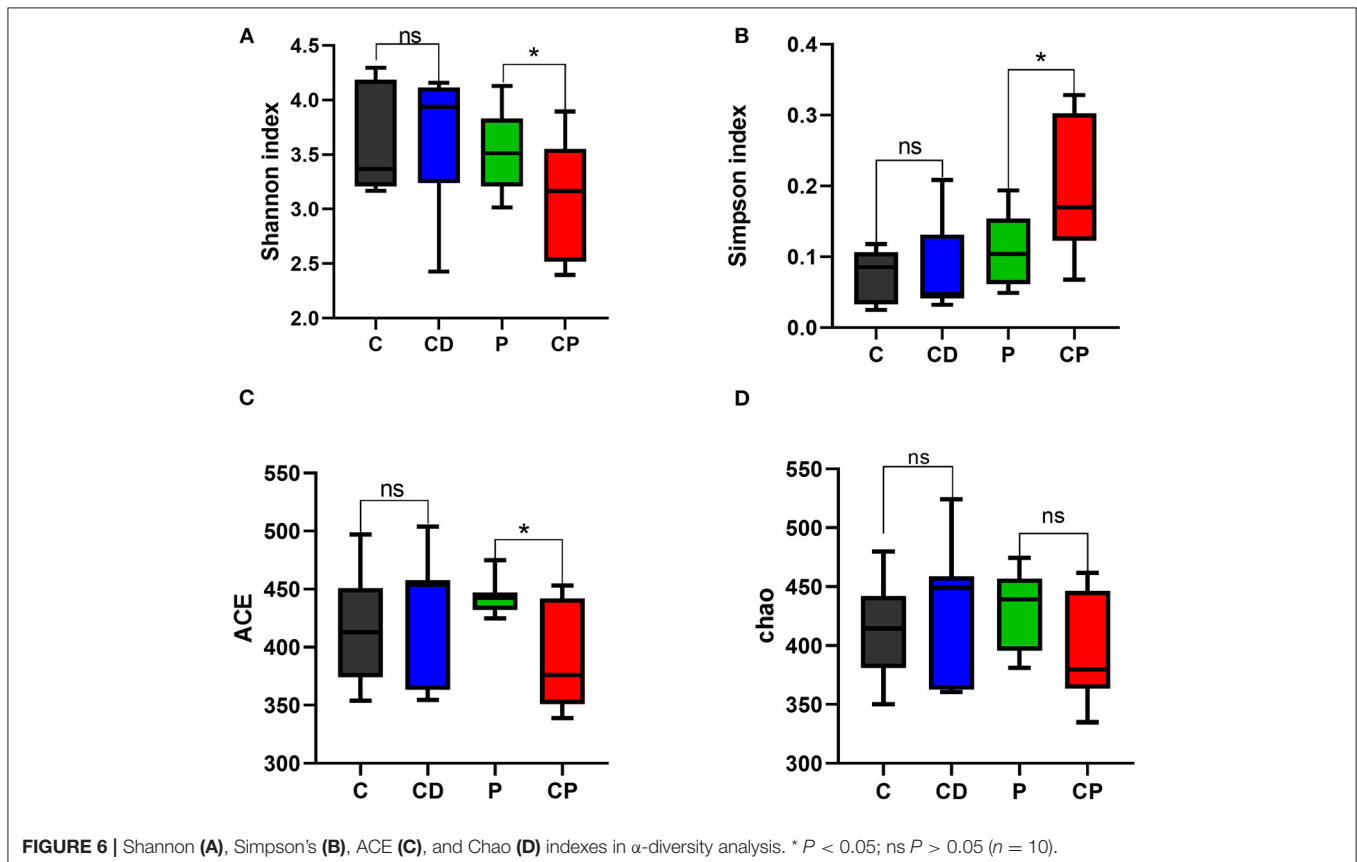
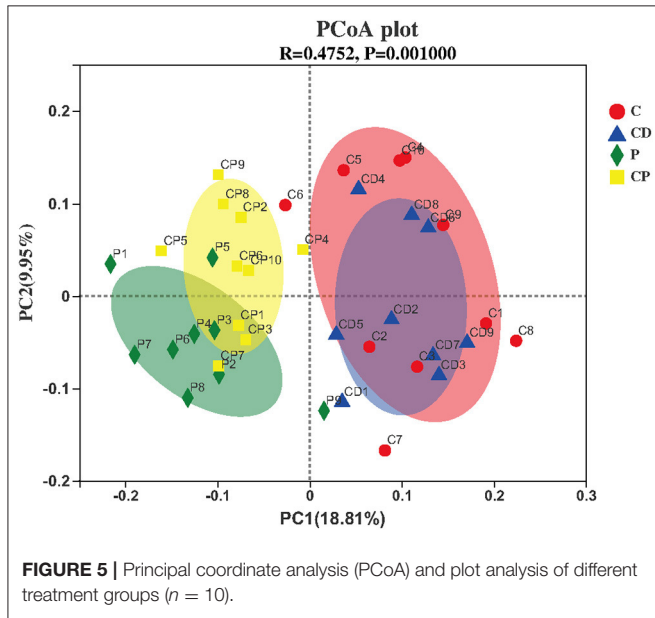


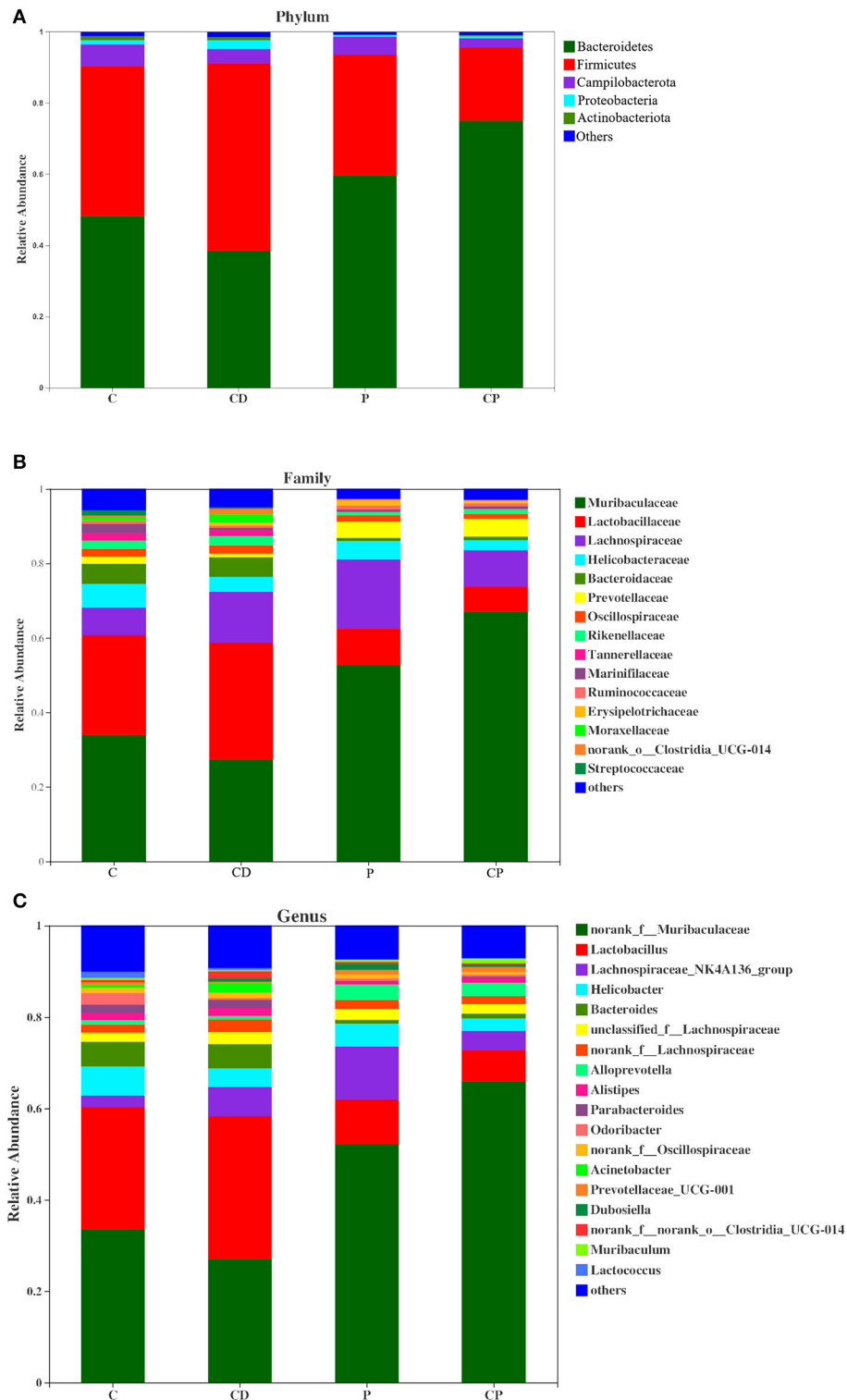
FIGURE 4 | The content of hepatic malonaldehyde (MDA) (A) and superoxide dismutase (SOD) (B) in liver ( $n = 8-10$ ). \*  $P < 0.05$ ; #  $P < 0.05$ ; and ns  $P > 0.05$ .

health parameters, microbial composition, and content of SCFAs. In healthy mice, *chenpi* supplement changed the accumulation of fat. In particular, *chenpi* powder can effectively reduce the weight

of abdominal adipose tissues, subcutaneous adipose tissue, and the serum level of TG. Other studies also have shown that *chenpi* can reduce the gain of body weight, organ weight, and accumulation of lipid (37). Obesity is closely related to hyperlipidemia, and reducing the content of serum triglyceride can effectively alleviate hyperlipidemia (38). There was no significant change in body weight and liver weight in our study, perhaps because the feeding time was not long enough. The effect of *chenpi* on the antioxidant activity of the liver was analyzed. MDA is the most frequently measured biomarkers of lipid peroxidation and oxidative stress that is considered hazardous to health (39). Oxidative damage can lead to a decrease in the content of SOD (40, 41). Here, the decoction of *chenpi* shows stronger antioxidant activity, which might be explained as more antioxidants are released from *chenpi* after decoction treatment using a high temperature (42).

*Chenpi* and its main active substances can affect the composition and richness of intestinal microorganisms. Hesperidin can increase the proportion of *Lactobacillus* in healthy mice. Citrus polymethoxyflavones can greatly enrich the bacterium *Bacteroides* in high-fat diet (HFD) mice (43–45). The abundance of *Proteobacteria* and the ratio of *Firmicutes* to *Bacteroidetes* were decreased by the *chenpi* extract in HFD mice. Although the addition of *chenpi* supplement did not significantly increase the abundance and diversity of intestinal microbiota in our study, it shows that *chenpi* powder has

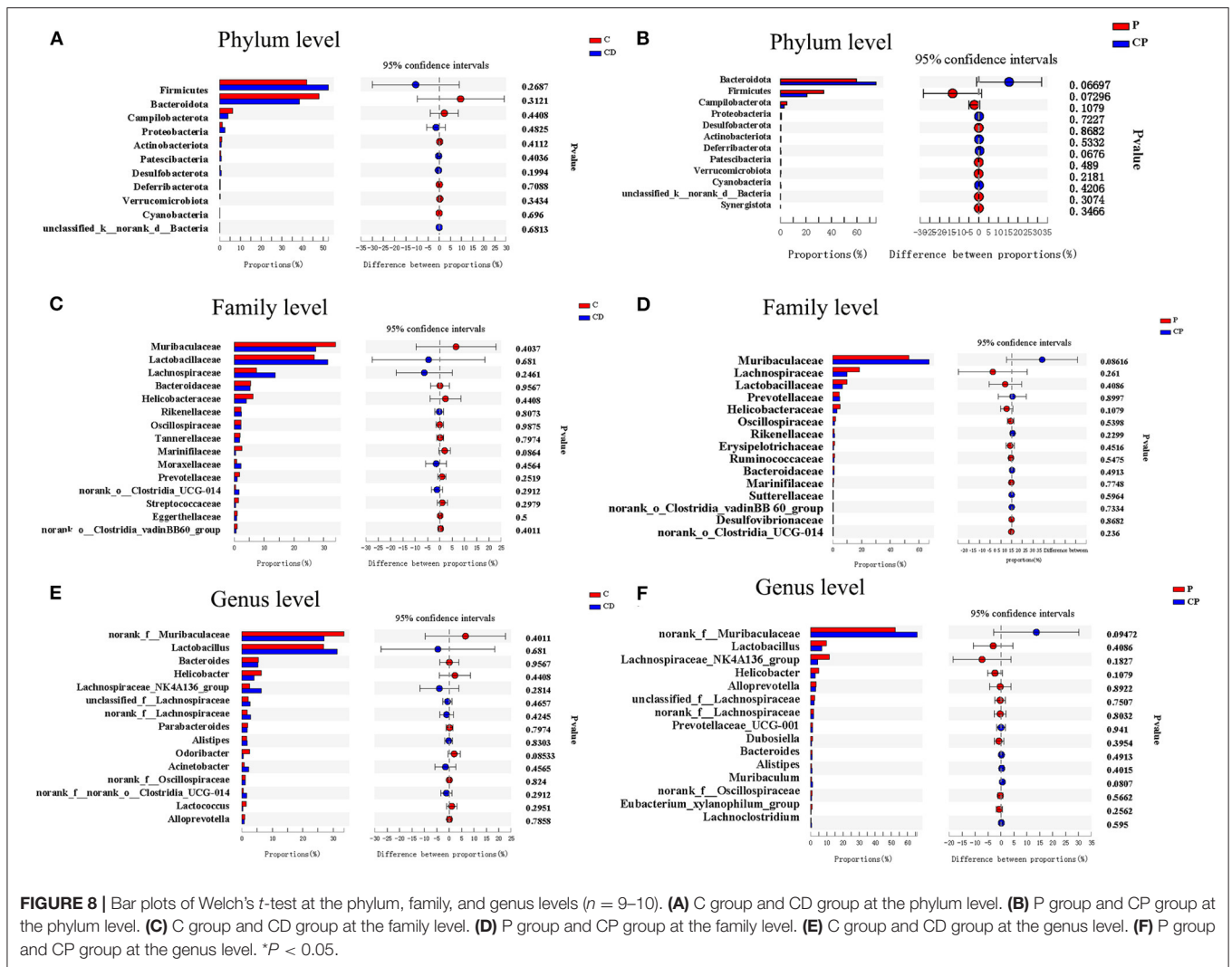




**FIGURE 7 |** Compositions of microbiota at the phylum (A), family (B), and genus levels (C) (n = 10).

a better tendency to increase the ratio of *Bacteroidetes* to *Firmicutes*. This may be because the decoction does not extract the active ingredients of *chenpi* very well and contains fewer

ingredients than *chenpi* powder. Although active compounds such as hesperidin, naringenin, and nobiletin can be detected in the water decoction of *chenpi*, some components cannot

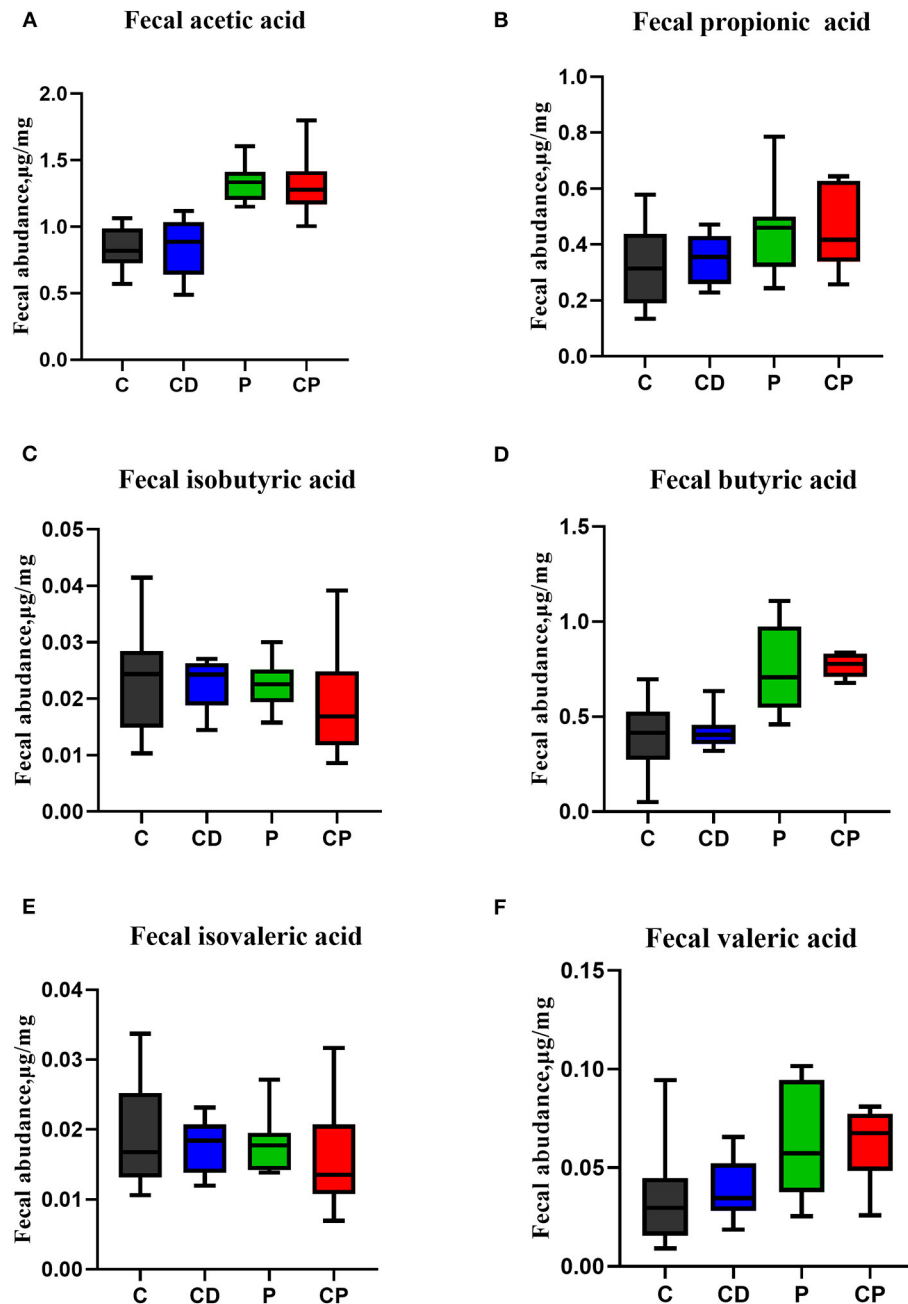


be fully and effectively extracted because of their poor water solubility (46). A study showed that the water solubility of 5-demethylnobiletin and hesperidin in *chenpi* was low (47, 48). ACE and Chao indexes reduced in the CP group. This may be related to the reduction in harmful bacteria. Studies show that the abundance of *Bacteroidetes* was reduced by 50%, but *Firmicutes* was increased about 18% (49, 50), the abundance ratio of *Bacteroidetes* to *Firmicutes* will decrease in fat mice compared to lean mice (51, 52), and our results are consistent with them. In the control group, *Muribaculaceae*, *Lactobacillaceae*, and *Lachnospiraceae* are the dominant strain at the family level. *Chenpi* powder increased the abundance of *Muribaculaceae* significantly at family and genus levels. A high abundance of *Muribaculaceae* is associated with longevity in mice (53). *Chenpi* decoction can increase the abundance of *Lactobacillaceae* that are intestinal beneficial bacteria (54). It has correlation coefficients between bacterial abundances and serum lipid oxidative. The correlations between the abundance of *Lactobacillaceae*, serum TG, and MDA levels were negative (55). *Chenpi* increased intestinal beneficial

bacteria and reduced microbial abundance associated with obesity. *Chenpi* powder is more outstanding in the regulation of intestinal microbiota.

The content of SCFAs is closely related to the diet structure. *Chenpi* contains not only many active ingredients but also a lot of dietary fiber. A fiber-rich diet can increase the content of SCFAs in mice. Dietary fiber can be fermented by colonic microbiota to produce SCFAs. Many studies have shown that a diet rich in dietary fiber can change the content of SCFAs. Passion fruit peel can increase the concentrations of butyrate and acetate in cecal content (56). Salami with citrus fiber increased the production of acetate, propionate, and butyrate (57). Dietary fibers from papayas promoted the production of SCFAs (58). Intestinal microorganisms are closely related to SCFAs. *Lachnospiraceae* plays an important role in the production of butyrate (59, 60). *Muribaculaceae* are helpful to the production of propionate (61). *Escherichia coli* could produce acetic acid (62). No significant changes in SCFAs were observed in our study, perhaps due to our shorter feeding cycle.





**FIGURE 9** | The effect of chenpi on short-chain fatty acids (SCFAs) in the feces ( $n = 9-10$ ). **(A)** Acetic acid, **(B)** propionic acid, **(C)** isobutyric acid, **(D)** butyric acid, **(E)** isovaleric acid, and **(F)** valeric acid.

In conclusion, daily consumption of *chenpi* has a certain effect on reducing weight and lipid. Compared with *chenpi* decoction, *chenpi* powder has a better effect in preventing obesity. *Chenpi* powder may be developed as supplementary functional food to prevent obesity in the future. In this study, we focused on the effect of different treatment methods of *chenpi* on healthy mice to predict the preventive effect on obesity. In the future, a high-fat model would be established to observe this effect in depth.

It is our next direction to research study to develop a variety of popular *chenpi* functional foods.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: PRJNA729616.

## ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use Committee of Hunan Agriculture University.

## AUTHOR CONTRIBUTIONS

JG: Conceptualization. YQ: methodology. YQ and JM: software. YQ, YS, and JG: writing-review and editing. YQ, CW, and ZG: visualization. GL, FF, and YS: supervision. YS: project administration and funding acquisition. All

authors contributed to the article and approved the submitted version.

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