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ORIGINAL ARTICLE

Polysaccharides and flavonoids from cyclocarya paliurus modulate gut microbiota and attenuate hepatic steatosis, hyperglycemia, and hyperlipidemia in nonalcoholic fatty liver disease rats with type 2 diabetes mellitus

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Abstract

Objective The prevalence of nonalcoholic fatty liver disease (NAFLD) with type 2 diabetes mellitus (T2DM) is increasing, which causes greater harm to human health. *Cyclocarya paliurus* (CP) has antihyperglycemic and antihyperlipidemic effects. Here, we investigated the effects of polysaccharides (CPP) and flavonoids (CPF) from CP on gut microbiota, hepatic steatosis, and metabolic parameters in high-fat diet (HFD)/streptozotocin (STZ)-induced NAFLD rats with T2DM.

Methods NAFLD/T2DM rats, which were induced by high-fat diet (HFD) for 8 weeks and a low dose of 25 mg/kg STZ, were treated with CPP (8 g/kg/d) or CPF (6 g/kg/d) for 12 weeks. The alterations to gut microbiota, hepatic steatosis, and metabolic parameters were measured.

Results Treatment of both CPP and CPF could improve liver steatosis, NAFLD activity score (NAS), hyperglycemia, and hyperlipidemia. Importantly, administration with both CPP and CPF led to the significant reversion of increased abundance of the pathogenic bacteria *Escherichia-Shigella* in NAFLD/T2DM rats; moreover, CPP supplement also dramatically increased the beneficial bacteria *Akkermansia* abundance, while CPF treatment significantly elevated the abundances of the beneficial bacteria *Romboutsia* and *Weissella*.

Conclusion Both CPP and CPF as prebiotics have the significant therapeutic effects on hepatic steatosis and metabolic abnormalities induced by HFD and STZ in rats at least partially by modulating gut microbiota.

Keywords Nonalcoholic fatty liver disease · Type 2 diabetes mellitus · Cyclocarya paliurus polysaccharides · Cyclocarya paliurus flavonoids · Metabolism · Gut microbiota

Introduction

The emergence of non-alcoholic fatty liver disease (NAFLD) and type 2 diabetes mellitus (T2DM) as a global epidemic is one of the major challenges to human health in the twenty-first century. NAFLD is now recognized as the most prevalent chronic liver disease worldwide, with a prevalence as high as 30% in the general population [1]. NAFLD includes a

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series of diseases ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), and to advanced cirrhosis and hepatocellular carcinoma [2]. It has been well-recognized that obesity, hyperlipidemia, diabetes mellitus, metabolic syndrome, and insulin resistance (IR) are considered as risk factors of NAFLD [3, 4]. T2DM is a complex metabolic disorder characterized by hyperglycemia, low-grade inflammation, IR, and β-cell failure and mainly affects glucose, lipid, and protein metabolism [5, 6]. It is apparent that NAFLD and T2DM share common risk factors, such as obesity, IR. The prevalence of NAFLD in the population of T2DM is increasing year by year, and has been found to be higher compared with that without T2DM [7, 8]. It should be noted that NAFLD with T2DM causes greater harm to health. People with NAFLD and T2DM are more likely to suffer from cardiovascular disease, chronic kidney disease, and carcinoma [3, 9].

Recent studies have shown that gut microbiota dysbiosis is associated with several non-communicable diseases such as obesity, diabetes, cardiovascular diseases, and NAFLD [10–12]. A direct involvement of gut microbiota in the development of NAFLD is suggested by the finding that NAFLD can be delivered to germ-free mice by fecal microbiota transplantation [13]. Imbalances in the structure of gut microbiota are related to gut barrier dysfunctions and cause insulin resistance and endotoxemia, which may finally lead to obesity and T2DM [14–18]. These results suggest that regulating gut microbiota could treat NAFLD and T2DM. At present, many of the anti-diabetic drugs currently in clinical use, though effectively treating symptoms, have several side effects including hepatic and renal lesions [19]. On the other hand, there is still no effective drugs for the treatment of NAFLD except for lifestyle changes, including healthy diet, weight loss, and exercise [20]. In addition, there is no consensus or therapeutic strategies for the management of NAFLD patients with T2DM.

Cyclocarya paliurus (CP) (Batal.) Iljinsk (family Cyclocaryaceae) is a plant with edible and medicinal value, which is grown in mountainous regions of Southern China. The leaves of CP have long been used as a dietary food and a traditional herbal medicine for the prevention or treatment of diabetes mellitus, hypertension, hyperliposis [21–23]. CP leaves contain a variety of biologically chemical components, including polysaccharides, flavonoids, coumarins, amino acids, sterols, and triterpenes [24], in which polysaccharides and flavonoids are recognized as the main bioactive components in CP [22, 25, 26]. It has been reported that polysaccharides and flavonoids from CP possess many bioactivities, such as anti-inflammatory, anti-hyperlipidemic, and anti-diabetic activities [23, 25–28].

However, to the best of our knowledge, no investigation has been performed to explore the protective effects of polysaccharides (CPP) and flavonoids (CPF) from CP on metabolic abnormalities and liver damage in NAFLD rats with T2DM (NAFLD/T2DM rats) induced by high-fat diet and streptozotocin (STZ). In the present study, we explored the effects of CPP and CPF on gut microbiota, hepatic steatosis, and metabolic parameters in NAFLD/T2DM rats.

Materials and methods

Preparation of CPP and CPF

The extraction of CPP was performed according to the method described previously [29]. Briefly, the air-dried and powdered leaves were soaked with 95% (v/v) ethanol for 12 h, and the mixture was filtered. The residues were dried in air and were boiled with distilled water at 95°C for 2 h (1:20, mg/mL). The above operation was repeated twice. Then, the mixture was filtered, and the filter liquor was retained. The filter liquor was concentrated by rotary evaporation at 60°C and then allowed to put overnight at 80% (v/v) ethanol concentration. The protein in the obtained CPP was removed with Savag method, and the CPP was dried under vacuum at −40°C. The CPP content was 79.6%, which was determined by phenol-sulfuric acid method [30] (Fig. 1).

Preparation of CPF was conducted according to the method described by Cheng et al. [31]. Briefly, CP leaves were pulverized and extracted with distilled water at 95◦C for 40min. After extraction, the extract was centrifuged at 4500×g for 15 min, and the above operation was repeated. The supernatants were combined and concentrated using a rotary evaporator. The residue was dissolved with deionized water, filtered by a 0.45-μm microfiltration membrane, and applied to a column (30×1.6 cm) of AB-8 resin. Finally, the effluent of ethanol solution was collected and concentrated, leading to the CPF extract. The CPF content was 84.3%, which was determined by aluminum chloride method [32] (Fig. 1).

Animal model and experiment design

A total of 60 male Sprague Dawley rats (weight, 180±20 g; age, 6 weeks) were purchased from the medical laboratory animal center of Guangdong (Guangzhou, China). All the rats were acclimatized under a temperature of 24±2°C, a relative humidity of 55±10%, and a 12 h light/dark cycle for 10 days before commencement of the animal experiment. All animal experiments were approved by the experimental animal ethics committee of Jinan University and was performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Jinan University.

After acclimation, these rats were randomly divided into the normal control group (NC, n=10) and the model group with both NAFLD and diabetes (NAFLD/T2DM Model, *n*=50). NAFLD/T2DM Model rats were induced by high-fat diet (HFD) (containing 34% fat, 2% cholesterol, 26%

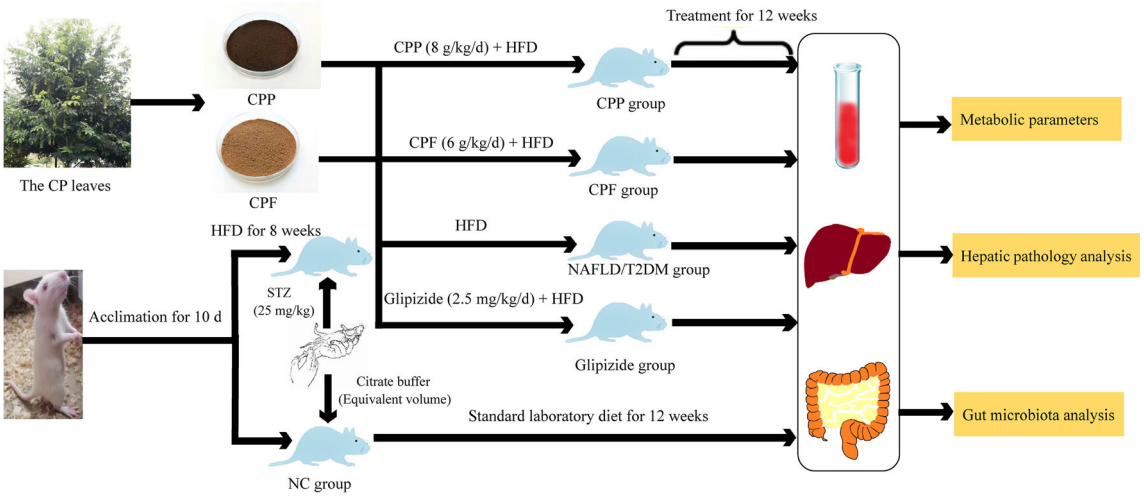

Fig. 1 CPP and CPF content

carbohydrate, 26% protein, and 12% basic feed (w/w)) for 8 weeks, followed by an intraperitoneal injection with streptozotocin (STZ) (25 mg/kg in citrate buffer). The rats with fasting glucose level higher than 11 mmol/L were considered as NAFLD/T2DM Model rats, and the NAFLD/T2DM Model rats with consecutive 10-day hyperglycemia (11 mmol/L or greater) were used for the experiment. Finally, 48 NAFLD/T2DM Model rats met the above experimental standard. In parallel, NC rats, which were fed a standard laboratory diet, were injected with an equal volume of citrate buffer solution.

The 48 NAFLD/T2DM Model rats were randomly divided into four groups: NAFLD/T2DM group (n=12), CPP group (n=12), CPF group (n=12), and Glipizide group (n=12), which were continuously fed with the HFD, and administrated by oral gavage once per day with distilled water, CPP (8 g/kg/d), CPF (6 g/kg/d), and Glipizide (2.5 mg/kg/d) (Glipizide extended release tablets**,** Glucotrol XL), respectively for the next 12 weeks. The NC group rats were fed with the standard laboratory diet for the next 12 weeks.

Observations on the general condition of the rats

The general condition of the rats was monitored daily, including physical activity, fur condition, water intake, food intake, urine output and survival condition. Body mass and food intake were determined weekly.

Analysis of metabolic parameters

At the end of the experiment, overnight-fasted rats were anesthetized by 1% pentobarbital solution (40mg/kg), and the blood samples were collected from abdominal aortic. The fasting blood glucose (FBG), alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl transpeptidase (GGT), total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), interleukin (IL)-6, and tumor necrosis factor (TNF)-α were analyzed using commercial assay kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturers' instructions. The fasting insulin (FINS) was measured with rat insulin kits (R&D Corporation, USA). The insulin resistant index (HOMA-IR) was also calculated by using the following formula:

$$\mathrm{HOMA-IR} = \mathrm{FBG\ (mmol/l)} \times \mathrm{FINS(\mu IU/ml)}/22.5$$

Analysis of hepatic pathology

At the end of the study, the rats were sacrificed to determine liver mass and liver mass index by using the following formula:

$$\text{live mass index} = \text{liver mass}/\text{body mass} \times 100\%$$

Then, the liver samples were immersed in 10% formalin neutral buffer solution for 48h, then processed routinely, embedded in paraffin, sectioned to 5 μm thickness and stained with hematoxylin and eosin (H&E). Then, we used Image-Pro Plus 6.0 software (*Media Cybernetics,* Rockville, MD, USA*)* to quantitatively analyze fat in liver [13]. The evaluation standard of NAFLD activity score (NAS) is shown in Table S1 [33, 34].

Gut microbiota analysis

At the end of the experiment, feces of rats were collected in sterilized plastic tubes and stored in a -80 °C until use. Total DNA of the samples was extracted using a HiPure Stool DNA Kits (Guangzhou Meiji biotechnology co. LTD, Guangzhou, China) following manufacturer's recommendations. The V3-V4 region of bacterial 16S rDNA gene was amplified using primers 341F (5'- CCT ACG GGN GGC WGC AG -3') and 806R (5'-GGA CTA CHV GGG TAT CTA AT-3').

Sequencing libraries were generated using two-step PCR amplification method. The second round of amplification products was purified using AMPure XP Beads (Beckman Coulter, USA) and quantified using a QuantiFluorTM fluoromete (Promega, USA). At last, the library was sequenced on IlluminaHiSeq 2500 platform (Illumina, USA).

Statistical analysis

Statistical analysis was performed using SPSS 21.0 software (IBM Corp., USA). Quantitative data were analyzed by independent-Samples *t* Test or one-way analysis of variance (ANOVA) followed by Mann-Whitney *U* test or Kruskal-Wallis H test. Correlation was evaluated by Pearson correlation co-efficient analysis. A difference with $p<.05$ was considered significant.

Results

Effects of CPP and CPF on general condition

The rats in the NAFLD/T2DM group showed sluggish action, irritability, polydipsia, *bulimia,* and polyuria, while those in the NC group did not. The above general condition was significantly improved in the CPP group, CPF group, and Glipizide group rats compared with the NAFLD/T2DM group rats.

A significant decrease in the body mass was observed in the NAFLD/T2DM group compared with the NC group. After treatment of both CPP and CPF, the rats showed a significant increase in the body mass compared with the NAFLD/T2DM group rat (Table 1). A significant increase in the body mass was also found in the Glipizide group compared with the NAFLD/T2DM group.

Effects of CPP and CPF on metabolic parameters

A significant increase in serum liver enzymes (ALT, AST, and GGT), serum lipid profile (TC, TG, LDL-C), glucose metabolism indices (FBG, FINS, and HOMA-IR), serum proinflammatory cytokines (TNF-α and IL-6) was observed in the NAFLD/T2DM group compared with the NC group. After treatment of both CPP and CPF, a remarkable decrease in the above metabolic parameters was found compared with the NAFLD/T2DM group. In addition, Glipizide treatment significantly lowered serum TG, glucose metabolism indices (FBG, FINS, and HOMA-IR), serum proinflammatory cytokines (TNF-α and IL-6), but had no effect on serum liver enzymes (ALT, AST, and GGT), and serum TC and LDL-C compared with the NAFLD/T2DM group (Table 1).

Effects of CPP and CPF on hepatic pathological changes

The rats in the NAFLD/T2DM group showed a significant increase in liver mass, liver mass index, liver fat content, and NAS compared with the NC rats, whereas both CPP and CPF intervention significantly decreased the above hepatic pathological parameters. Glipizide treatment also lowered liver mass index, but had no significant influence on liver mass, liver fat content, and NAS compared with the NAFLD/T2DM group (Table 1 and Fig. 2).

Effects of CPP and CPF on gut microbiota composition

The structural changes of gut microbiota are crucial in the pathogenesis of obesity and some other metabolic diseases. To assess the changes of gut microbiota composition after treatment of both CPP and CPF in NAFLD/T2DM rats, 16S rDNA gene sequences from bacterial populations of intestinal contents were analyzed. At the phylum level, the 5 most abundant microbiota in all groups included *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Verrucomicrobia*, and *Proteobacteria* (Fig. 3A). The relative abundances of *Firmicutes* (51.1%) and *Bacteroidetes* (0.3%) decreased significantly, while the relative abundance of *Proteobacteria* (29.3%) increased significantly in the NAFLD/T2DM group compared with the NC group (Fig. 3B–D). CPP treatment significantly decreased the relative abundances of *Firmicutes* (24.0%) and *Proteobacteria* (1.8%) (Fig. 3B and D), and increased the relative abundance of *Verrucomicrobia* (69.4%) (Fig. 3E), whereas CPF treatment remarkably increased the abundances of *Firmicutes* (73.4%) and *Bacteroidetes* (1.9%) compared with the NAFLD/T2DM group (Fig. 3B and C). In addition, the ratio of *Firmicutes* to *Bacteroidetes* increased significantly in the NAFLD/T2DM group compared with the NC group, whereas treatment of both CPP and CPF led to the reduction of this ratio but this reduction was not significant compared with the NAFLD/T2DM group (Fig. 3F). In the Glipizide group, the variation of the gut microbiota abundance was similar to the CPF group (Fig. 3B and Table S2).

At the genus level, the composition of the bacteria was substantially changed (Fig. 4A). The relative abundances of *Ruminococcaceae_UCG-005* (0.6%), *Lactobacillus* (4.3%), *Weissella* (1.0%), *Romboutsia* (4.1%) decreased significantly (Fig. 4B–E), while the relative abundances of *Escherichia-Shigella* (22.0%), *Collinsella* (6.0%), and *Blautia* (6.6%) increased markedly in the NAFLD/T2DM group compared with the NC group (Fig. 4–H). CPP treatment decreased the relative abundance of *Escherichia-Shigella* (1.0%) and increased the relative abundance of *Akkermansia* (45.3%) significantly (Fig. 4F and I), while CPF treatment decreased the relative abundance of *Escherichia-Shigella* (4.6%) (Fig. 4F) and increased the relative abundances of *Romboutsia* (10.9%) (Fig. 4E) and

Table 1 Metabolic characteristics and hepatic pathological changes in laboratory rats

Items	NC (*n*=9)	NAFLD/T2DM (*n*=10)	CPP (*n*=9)	CPF (*n*=9)	Glipizide (*n*=9)
Body mass (g)					
Mean ± SD	589.1±70.2	429.4±55.9**	500.1±62.4#	498.3±25.5 ##	509.8±67.0 #
Median (IQR)	551.0 (532.0-643.0)	433.0 (372.4-479.3) **	505.0 (438.5-552.0) #	510.0 (472.5-520.0) ##	513.0 (439.5-571.0) #
Serum liver enzymes (U/L)					
ALT					
Mean ± SD	46.0±7.6	270.3±107.6 **	157.5±54.3 #	66.5±23.6 ##	210.8±30.3
Median (IQR)	43.3 (38.9-53.5)	248.8 (176.9-339.9) **	156.3 (128.6-174.4) ##	75.2 (40.6-88.6) ##	208.1 (183.6-228.2)
AST					
Mean ± SD	106.3±18.7	542.6±179.9**	196.4±78.3##	173.6±63.0##	564.7±92.6
Median (IQR)	109.9(89.4-125.8)	566.1(335.9-723.1) **	162.1(139.7-266.0) ##	156.5(137.3-200.0) ##	540.0(490.0-618.9)
GGT					
Mean ± SD	36.0±11.1	160.7±57.1**	86.3±17.8##	67.0±18.0##	154.4±44.1
Median (IQR)	33.2 (27.9-44.0)	142.9 (114.0-221.5) **	81.2 (71.8-97.5) ##	63.7 (56.1-82.6) ##	159.4 (115.3-189.6)
Serum lipid profile (mmol/L)					
TC					
Mean ± SD	1.6±0.6	7.1±4.0 **	2.7±0.9 ##	2.5±0.5 ##	6.8±2.7
Median (IQR)	1.6(1.0-2.1)	6.6(3.5-9.9) **	2.3(2.2-3.0) ##	2.3(2.2-3.0) ##	5.9(4.4-9.0)
TG					
Mean ± SD	0.8±0.6	7.6±2.2 **	1.0±0.8 ##	1.0±0.4 ##	4.3±1.2 ##
Median (IQR)	0.6(0.4-1.3)	7.7(5.7-9.5) **	0.6(0.6-1.0) ##	1.1(0.6-1.3) ##	4.0(3.2-5.3) ##
LDL-C					
Mean ± SD	0.7±0.4	2.5±1.5 **	1.2±0.2 #	1.2±0.5 #	2.2±1.5
Median (IQR)	0.6(0.4-1.1)	2.2(1.3-3.5) **	1.2(0.9-1.3) ##	1.1(0.8-1.7) #	2.0(0.9-3.2)
Glucose metabolism indices					
FBG (mmol/L)					
Mean ± SD	4.6±1.2	18.5±1.9 **	11.9±1.2 ##	13.8±2.1 ##	13.5±1.9 ##
Median (IQR)	4.3 (3.7-5.5)	18.5 (17.1-19.0) **	11.9 (11.0-13.0) ##	14.0 (12.4-15.7) ##	13.1 (12.3-15.6) ##
FINS (μIU/ml)					
Mean ± SD	15.9±2.9	49.7±4.2 **	21.3±3.8 ##	25.5±4.1##	25.3±3.2 ##
Median (IQR)	15.3 (13.2-18.7)	49.7 (46.1-53.3) **	19.7 (18.4-25.3) ##	25.3 (21.5-29.0) ##	25.8 (22.9-27.0) ##
HOMA-IR					
Mean ± SD	3.3±1.0	40.9±5.4 **	11.3±2.2 ##	15.7±3.7 ##	15.1±2.2 ##
Median (IQR)	3.1 (2.4-4.1)	38.7 (37.5-45.4) **	11.5 (9.1-13.5) ##	15.5 (14.8-16.5) ##	14.7 (13.4-17.0) ##
Serum proinflammatory cytokines (pg/ml)					
TNF-α					
Mean ± SD	38.0±15.5	128.5±18.8 **	60.5±13.5 ##	66.3±18.0 ##	93.3±10.8 ##
Median (IQR)	35.5 (24.1-52.6)	124.4 (119.0-136.5) **	63.0 (48.5-70.0) ##	59.5 (54.8-85.9) ##	91.8 (84.7-105.0) ##
IL-6					
Mean ± SD	61.1±22.7	840.3±119.2 **	538.2±157.5 ##	125.5±42.8 ##	317.2±45.9 ##
Median (IQR)	50.4 (45.7-85.0)	822.4 (769.1-965.4) **	501.8 (390.0-684.7) ##	106.5 (93.9-148.3) ##	319.8 (285,9-355.4) ##
Hepatic pathological indices					
Liver mass (g)					
Mean ± SD	12.6±1.8	29.2±4.5 **	18.1±3.0 ##	15.6±1.9 ##	27.8±2.5
Median (IQR)	13.2 (10.5-14.3)	28.4 (26.3-33.6) **	17.8 (15.9-20.6) ##	16.1 (13.8-16.9) ##	28.5 (25.4-30.0)
Liver mass index (%)					
Mean ± SD	2.2±0.4	6.8±1.0 **	3.7±0.9 ##	3.1±0.4 ##	5.5±0.6 ##
Median (IQR)	2.0 (1.9-2.5)	6.8 (5.9-7.8) **	3.8 (2.8-4.2) ##	3.2 (2.8-3.5) ##	5.3 (5.0-5.9) ##

Table 1 (continued)

Items	NC (*n*=9)	NAFLD/T2DM (*n*=10)	CPP (*n*=9)	CPF (*n*=9)	Glipizide (*n*=9)
Liver fat content (%)					
Mean ± SD	1.0±1.3	66.4±9.9 **	22.5±7.0 ##	26.8±8.2 ##	59.0±9.1
Median (IQR)	0.0 (0.0-2.0)	64.1 (58.4-76.5) **	23.8 (17.9-28.2) ##	27.6 (21.1-34.6) ##	58.7 (53.4-67.1)
NAS					
Mean ± SD	0.1±0.1	6.5±0.3 **	3.1±0.4 ##	2.0±0.5 ##	6.1±0.7
Median (IQR)	0.1 (0.0-0.1)	6.5 (6.2-6.9) **	3.2 (2.8-3.4) ##	2.1 (1.5-2.4) ##	6.0(5.6-6.7)

*p<0.05, **p<0.01 vs NC group; #p<0.05, ##p<0.01 vs NAFLD+T2DM group. The data are shown as mean and median (interquartile range, IQR). *NC* normal control, *NAFLD/T2DM* NAFLD with T2DM, *CPP* cyclocarya paliurus polysaccharides, *CPF* cyclocarya paliurus flavonoids, *SD* standard deviation, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *GGT* γ-glutamyl transpeptidase, *TC* total cholesterol, *TG* triglyceride, *LDL-C* low-density lipoprotein-cholesterol, *FBG* fasting blood glucose, *FINS* fasting insulin, *HOMA-IR* = (fasting glucose × fasting insulin)/22.5, *TNF-α* tumor necrosis factor α, IL-6 interleukin-6, NAS NALFD activity score

Weissella (4.2%) (Fig. 4D) significantly compared with the NAFLD/T2DM group. In the Glipizide group, the relative abundance of *Escherichia-Shigella* (2.5%) decreased markedly (Fig. 4F) and the relative abundances of *Lactobacillus* (10.4%) and *Romboutsia* (6.4%) and *Dubosiella* (8.1%) (Fig. 4C, E, and J) increased significantly (Table S3).

Correlation between improvements of metabolic indices as well as hepatic pathology and the changes in gut bacterial genera induced by CPP and CPF

We examined whether the improvements of metabolic indices and hepatic pathology were correlated with the alternations of bacterial genera induced by CPP and CPF. Pearson correlation analysis showed that the alternation of Akkermansia level was negatively correlated with the main metabolic and hepatic pathological indices, whereas Escherichia-Shigella level was positively correlated with most of the above results after CPP treatment. Moreover, other CPP-modulated genera, such as *Blautia, Lactobacillus, Dubosiella, Collinsell, Romboutsia, Weissella, Aerococcus, and Ruminococcaceae_UCG-005* also contributed to the changes in a few indices of the above results (Fig. 5A). On the other hand, the level of Romboutsia was negatively correlated with most of the metabolic and hepatic pathological parameters, whereas Escherichia-Shigella, Collinsell, Dubosiella, and Aerococcus were positively correlated with the partial above indices after CPF treatment. In addition, *Blautia, Lactobacillus, Akkermansia, Weissella,* and *Ruminococcaceae_UCG-005* enriched by CPF were also negatively associated with some serum liver enzymes and lipid profile (Fig. 5B).

Discussion

In the present study, a rat model of NAFLD/T2DM was induced by HFD for 8 weeks and a low dose of 25 mg/kg STZ,

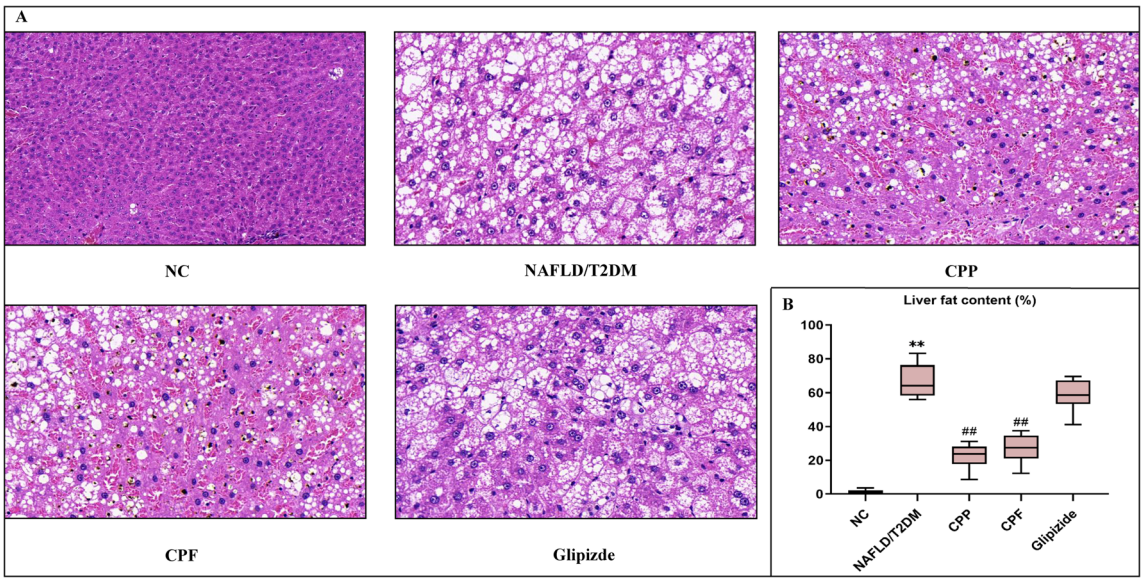

Fig. 2 Glipizide treatment also lowered liver mass index

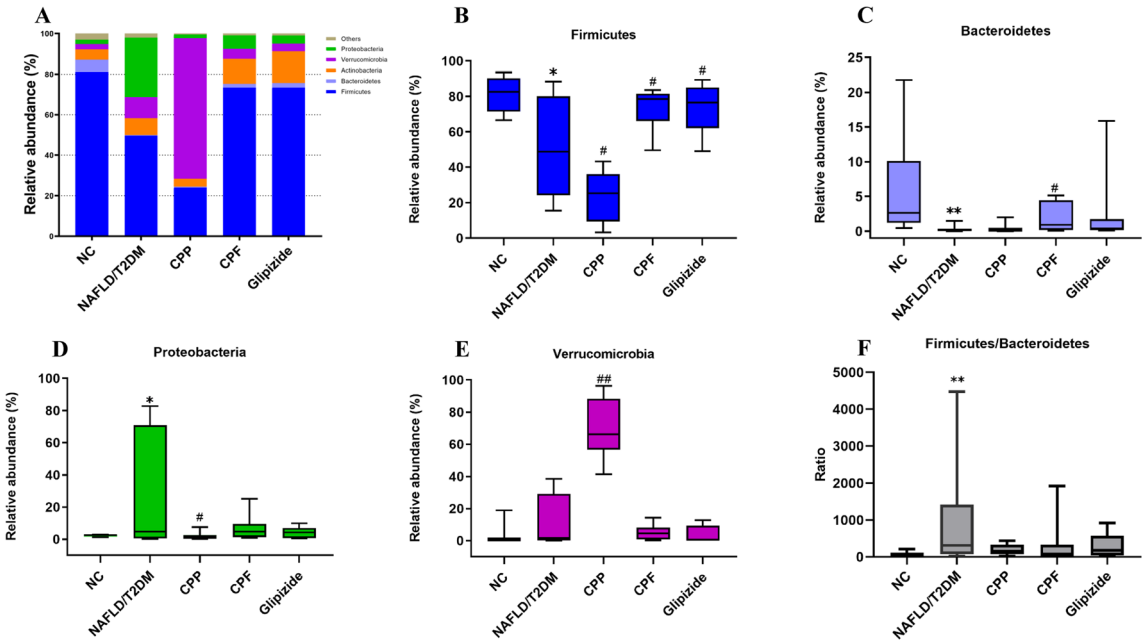

Fig. 3 The 5 most abundant microbiota in all groups

which reveals similar metabolic characteristics and liver damage of NAFLD with T2DM in humans [35, 36]. Treatment with both CPP and CPF significantly improved body mass, liver enzymes (ALT, AST, and GGT), blood lipids (TC, TG, and LDL-C), glucose metabolism parameters (FBG, FINS, and HOMA-IR), proinflammatory cytokines (TNF-α and IL-6), and hepatic pathological parameters (liver mass, liver mass index, liver fat content, and NAS), while Glipizide treatment elevated body mass and only reduced glucose metabolism parameters (FBG, FINS, and HOMA-IR) and proinflammatory cytokines levels (TNF-α and IL-6) in the NAFLD/T2DM rats. These results suggest that the protective effects of both CPP and CPF on NAFLD with T2DM in rat models are superior to Glipizide. Similar to our results, Lin et al. found

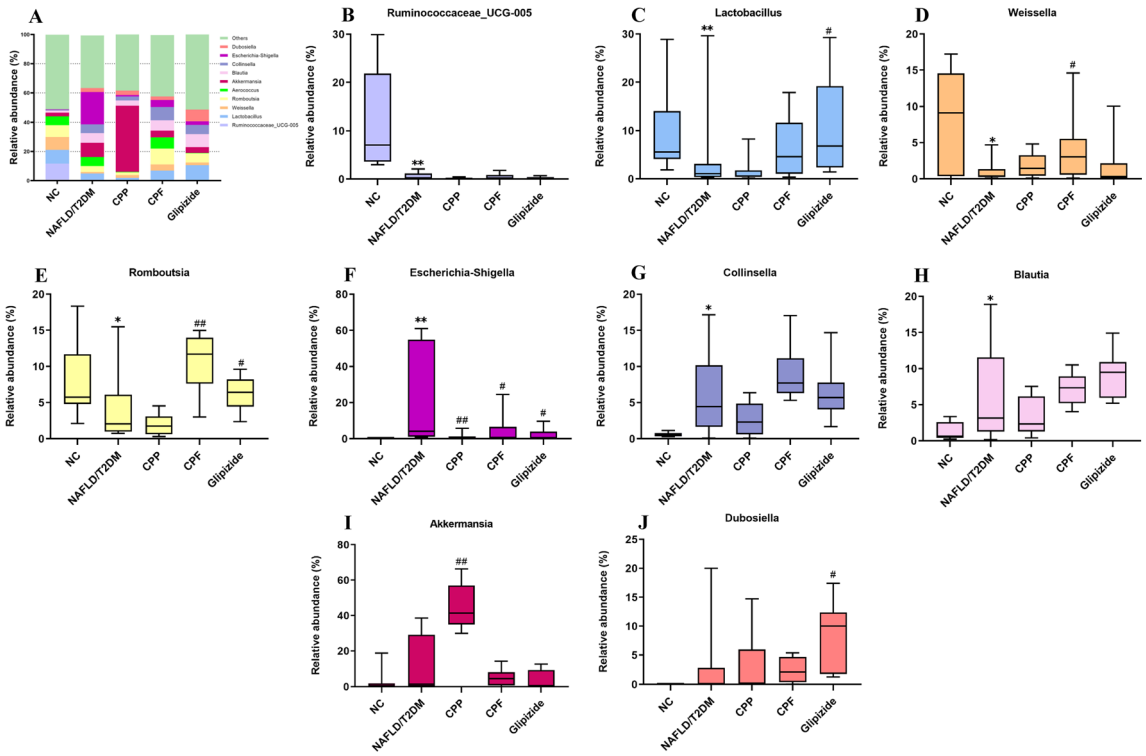

Fig. 4 The composition of the bacteria

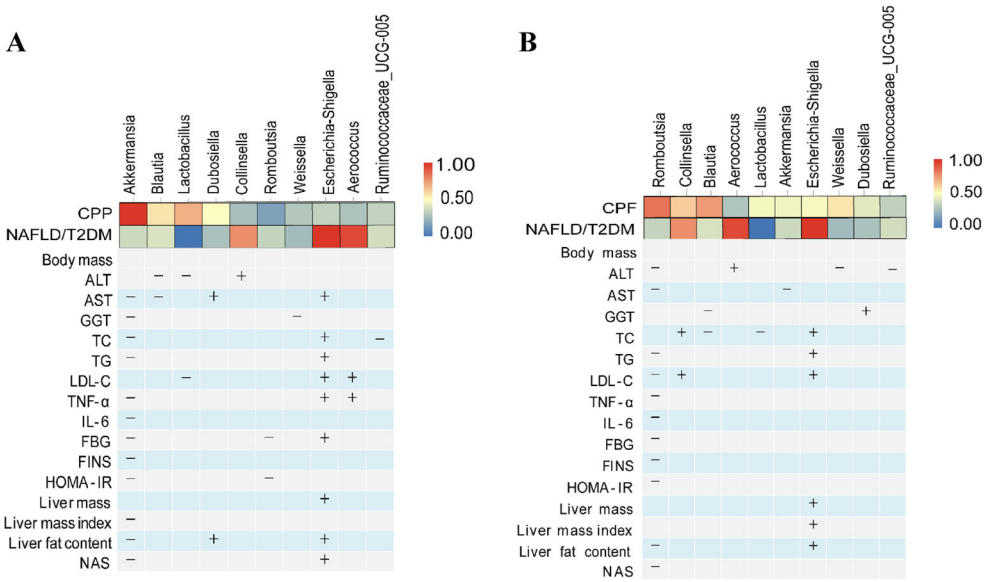

Fig. 5 CPP-modulated genera

that chloroform extract of CP markedly decreased the levels of serum liver enzymes (ALT, AST, and ALP), blood lipids (TC and TG) and liver lipids (TC and TG), and serum and liver TNF- α in NAFLD rats [37]. These effects were partially through decreasing serum NEFAs which might lead to a decrease in the amount of liver lipid intake, as well as suppressing hepatic lipid de novo synthesis. CPP and CPF also ameliorated HFD-induced hepatic oxidative stress and inflammation, leading to block the development of NAFLD.

The mechanisms responsible for the protective effect of CP on NAFLD and T2DM have attracted much attention. Accumulating evidence has indicated that gut microbiota dysbiosis is correlated with the pathogenesis of NAFLD, T2DM and obesity [23, 38–40], thus speculating that the therapeutic role of CP in metabolic diseases might be partially attributed to the alteration of gut microbes. It has been shown that a higher ratio of Firmicutes to Bacteroidetes is observed in obese individuals than lean individuals [40], and this ratio is significantly reduced after CPF treatment in a high-fat diet-induced obesity mouse model [31].

In the current study, at the phyla level, oral administration of CPP dramatically elevated the abundance of *Verrucomicrobia* and lowered the abundances of *Proteobacteria* and *Firmicutes* in the NAFLD/T2DM rats, and the ratio of *Firmicutes* to *Bacteroidetes* also showed a decrease trend after CPP treatment; on the other hand, CPF treatment significantly recovered dysbiosis of *Firmicutes* and *Bacteroidetes* and reduced the ratio of *Firmicutes* to *Bacteroidetes* (although there was no significant difference in this ratio) in the NAFLD/T2DM rats. At the genus level, oral administration with both CPP and CPF led to the significant reversion of increased abundance of *Escherichia-Shigella* induced by HFD and STZ in rats; in addition, CPP supplement dramatically increased the abundance of *Akkermansia*, while CPF treatment led to a significant increase in the abundances of *Romboutsia* and *Weissella*. Further pearson linear correlation analysis showed that the significant increase in *Akkermansia* abundance and the significant decrease in *Escherichia-Shigella* abundance induced by CPP treatment were associated with the improvements of the main metabolic and hepatic pathological indices; on the other hand, elevated *Romboutsia* level and reduced *Escherichia-Shigella* level after CPF treatment were correlated with the alleviation of most abnormal metabolic and hepatic pathological parameters in NAFLD/T2DM rats. Besides, Glipizide treatment led to the increased abundances of *Lactobacillus, Romboutsia*, and *Dubosiella* and the decreased abundance of *Escherichia-Shigella*.

Consistent with our results, the report by Yao et al. indicated that CPP treatment alleviates blood glucose, blood lipid, and HOMA-IR index by increasing the short-chain fatty acids (SCFAs)-producing gut bacteria in rats with T2DM [41]; Li et al. showed that CPP treatment increased the beneficial bacteria genus *Ruminococcaceae UCG-005* abundance, which in turn attenuated FBG and HOMA-IR in type 2 diabetic rats [38]; Bai et al. showed that treatment of flavonoids of Quzhou Fructus Aurantii extract significantly reduced obesity, inflammation and liver steatosis by the reduction of *Firmicutes* to *Bacteroidetes* ratio, the increase in genera *Akkermansia* and *Alistipes*, and the decrease in genera *Dubosiella*, *Faecalibaculum*, and *Lactobacillus* in HFD-induced obesity mouse model [42]; the report by Li et al. revealed that Silybin administration showed protective effects against high-fat diet-induced obesity, insulin resistance, and liver steatosis in mice, which was associated with lowering the *Firmicutes* to *Bacteroidetes* ratio and increasing the abundances of SCFA-producing bacteria (*Blautia, Bacteriodes, and Akkermensia*) [43].

It has been shown that *Escherichia-Shigella, Aerococcus, Collinsella,* and *Dubosiella* may contribute to the development of metabolic diseases such as obesity, NAFLD, and T2DM [42, 44–49]. On the other hand, it has been accepted that decreased abundances of some beneficial bacteria in gut, such as *Ruminococcaceae_UCG-005, Lactobacillus, Akkermansia, and Blautia,* are associated with obesity, NAFLD, and T2DM [42, 43, 50]. These beneficial bacteria can produce SCFAs by metabolizing polysaccharides, and in turn maintain the integrity of the intestinal mucosal barrier [38, 51, 52]. SCFAs have a variety of physiological functions, including shaping the gut environment, influencing the physiology of the colon, being utilized as energy sources by host cells and intestinal microbiota, and participating in different host-signaling mechanisms [53, 54]. *Akkermansia* can also degrade mucin, thereby protecting the intestinal mucosal barrier and reducing protein deposition [55]. *Weissella* species, which are Gram-positive coccobacilli, are potential probiotics [56, 57]. The genus *Romboutsia*, which also are Gram-positive organisms, are in the gut of healthy humans and rats [58–60].

Taken together, our findings suggest that both CPP and CPF as prebiotics could partially recover the gut microbiota equilibrium, especially with CPP inhibiting the growth of *Escherichia-Shigella* and dramatically increasing *Akkermansia* population, and with CPF restraining the growth of *Escherichia-Shigella* and enhancing the abundances of *Romboutsia* and *Weissella* in NAFLD/T2DM rat model. Such changes in the composition of the gut microbiota in turn improve liver steatosis, hyperglycemia, hyperlipidemia, insulin resistance, and inflammation induced by HFD and STZ in rats.

In conclusion, to the best of our knowledge, this study is the first report that demonstrated administration with both CPP and CPF had the significant therapeutic effects on liver steatosis and metabolic abnormalities induced by HFD and STZ in rats. The mechanism responsible for the effects may be at least partially correlated with modulating gut microbiota composition, as indicated by inhibiting the growth of pathogenic bacteria *Escherichia-Shigella* and discrepantly expanding the abundances of beneficial bacteria *Akkermansia*, or *Romboutsia* and *Weissella* by CPP and CPF treatment, respectively.

Abbreviations NAFLD, Nonalcoholic fatty liver disease; T2DM, Type 2 diabetes mellitus; CP, Cyclocarya paliurus; CPF, Cyclocarya paliurus polysaccharides; CPP, Cyclocarya paliurus flavonoids; HFD, High-fat diet; STZ, Streptozotocin.; NAS, NALFD activity score; NASH, Nonalcoholic steatohepatitis; IR, Insulin resistance; FBG, The fasting blood glucose; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; GGT, γ-glutamyl transpeptidase; TC, Total cholesterol; TG, Triglyceride; LDL-C, Low-density lipoprotein cholesterol; IL, Interleukin; TNF, Tumor necrosis factor; FINS, The fasting insulin; HOMA-IR, The insulin resistant index

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Declarations

Ethics approval All animal experiments were approved by the experimental animal ethics committee of Jinan University and was performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Jinan University (2019021101).

Conflict of interest The authors declare no competing interests.

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