

Protocatechuic Acid as a Phenolic Intermediate to Ameliorate Non-alcoholic Fatty Liver Disease by Inhibiting Enterococcus Faecalis in Mice

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Research

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Abstract

Objective: To assess the protective effects and mechanisms of cyanidin 3-glucoside (C3G) and its major phenolic metabolites including protocatechuic acid (PCA), phloroglucinaldehyde (PGA), vanillic acid (VA) and ferulic acid (FA) on non-alcoholic fatty liver disease (NAFLD).

Design: First, C57BL/6J mice were fed a low-fat diet (LFD), high-fat diet (HFD) only, or HFD containing 0.4% C3G, PCA, PGA, VA or FA for 12 weeks to understand their protective effects against NAFLD. Next, the dose-dependent effect and mechanism of PCA against NAFLD were studied in a mouse model by supplementing 0.025% or 0.1% PCA in HFD. Eventually, the bacteriostatic effect of PCA on *Enterococcus faecalis* was confirmed *in vitro*, and the role of *Enterococcus faecalis* in NAFLD was discovered by fecal microbiota transplantation (FMT) experiment.

Results: As the major phenolic metabolite of C3G, PCA dose-dependently reduced intraperitoneal and hepatic fat deposition, with alleviated insulin resistance, hepatic inflammation and lipid peroxidation. Characterization of gut microbiota indicated that PCA decreased the Firmicutes/Bacteroidetes ratio mainly by reducing the relative abundance of *Roseburia*, *Intestinibacter* and *Enterococcus*. Lipidomics and correlation analysis revealed that *Enterococcus* had a significant correlation with the changed lipid metabolites by PCA. *In vitro* bacteriostatic experiment showed that PCA has a direct inhibitory effect against *Enterococcus faecalis*, which has been proved to accelerate NAFLD by fecal microbiota transplantation.

Conclusion: This study demonstrated the first time that PCA can protect against NAFLD through inhibiting *Enterococcus faecalis*.

1 Introduction

The occurrence and development mechanism of non-alcoholic fatty liver disease (NAFLD) are complicated, although primarily based on the correlations between genetic and nutritional factors including lipids metabolism disorder, insulin resistance and intestinal microecological imbalance^[1]. Since metabolic syndromes are popular in individuals with over-nutrition, gut microbiota are considered to play an important role in NAFLD, such as short-chain fatty acids (SCFAs)-producing bacteria^[2,3] and microbiota belonging to Lactobacillales^[4]. Recent studies have suggested that *Enterococcus faecalis* (*E. faecalis*), an opportunistic pathogen belonging to Lactobacillales, has the potential to induce liver steatosis since it can secrete cytotoxin^[5], and has capability of metabolizing trimethyllysine into N,N,N-trimethyl-5-aminovaleric acid, which can reduce carnitine synthesis to decrease oxidation of fatty acids^[6]. Considering that, changes of gut microbiota become the prime concern in understanding the pathogenesis and prevention of NAFLD.

Polyphenols offer a great potential as an alternative therapy for NAFLD due to their biological functions such as antioxidant^[7], anti-inflammatory^[8], and anti-bacterial^[9] in recent years. Our previous study has suggested that cyanidin 3-glucoside (C3G), one of the most common anthocyanins in plant, has the potential to ameliorate inflammation and oxidative stress in NAFLD mice^[10], by modulating gut microbiota^[11]. However, after ingestion, C3G can be rapidly degraded into phenolic acids, which may take the primary responsibility for the biological function^[12], and protocatechuic acid (PCA), phloroglucinaldehyde (PGA), vanillic acid (VA),

ferulic acid (FA) and their derivatives have been proved as the major phenolic metabolites of C3G in blood circulation [13, 14]. Based on the antioxidant and anti-inflammatory activities, PCA, PGA, VA and FA are suggested to be the potential bioactive phenolic metabolites of C3G in our recent study [15]. Therefore, this study aimed to investigate the protective effects of C3G, PCA, PGA, VA and FA against NAFLD, and challenged to clarify the protective mechanisms of PCA, a typical bioactive metabolite, focusing on regulation of gut microbiota in a high fat diet-induced mouse model.

2 Materials And Methods

2.1 Diets and reagents

Both low-fat diet (LFD, D12450J) and high-fat diet (HFD, D12492) were purchased from Research Diets Inc. (New Brunswick, NJ, USA), and the diet composition was shown in Supplemental Table 1 (also available on <http://researchdiets.com/>). C3G, PCA, VA, and FA were provided by Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). PGA was purchased from Sigma-Aldrich (St. Louis, MO, USA). *Enterococcus faecalis* (ATCC 29212) was provided by South China Agricultural University.

2.2 Mouse model and experimental design

The experimental procedures were approved by the Hunan Agricultural University Institutional Animal Care and Use Committee (Permission No. 2018001). Mice and sterilized poplar bedding were purchased from Hunan Slake Jingda Laboratory Animal Co., Ltd. (License No. SCXK-Xiang 2016-0002, Changsha, Hunan, China). Mice were housed separately in cages with sterile environment under controlled temperature (23.5 °C) and light (12 h light/day), and had free access to feed and water. In the first experiment, twenty-eight C57BL/6J mice (SPF class, male, 6 weeks of age) were randomly allocated into 7 groups (n = 4) after acclimatization for 1 week. Mice were then fed a LFD, a HFD, or a HFD containing 0.4% (w/w) of C3G, PCA, PGA, VA, or FA for 12 weeks, respectively. The dosages of C3G and its phenolic metabolites were based on our previous study [10]. In the second experiment, twenty-four C57BL/6J mice (SPF class, male, 4 weeks of age) were randomly divided into 4 groups (n = 6) after acclimatization for 1 week, and then fed a LFD, a HFD, or a HFD containing 0.025% or 0.1% (w/w) of PCA for 12 weeks, respectively.

2.3 Bacteriostatic experiment

E. faecalis was cultured with brain heart infusion (BHI) medium and identified by comparing with sequence of *E. faecalis* ATCC 29212 on National Center for Biotechnology Information (NCBI) (Supplemental Fig. 2). The bacteriostatic effect of PCA (0.355–45.455 mM) was tested by detecting the optical density (OD) value under 600 nm after 24 h incubation. The calculation of Transmittance accords to the following formula:

$$\text{Transmittance (\%)} = \frac{[(\text{OD of Negative control} - \text{OD of Blank control}) - (\text{OD of Test} - \text{OD of Positive control})]}{(\text{OD of Negative control} - \text{OD of Blank control})}$$

The minimum inhibitory concentration (MIC) depends on all value of Transmittance > 95 (only valid when negative controls were turbid) [16, 17]. After that, “x” was converted to “log₁₀(x)” to achieve fitting curve

between Concentration (x, mM) and Transmittance (y, %). And the theoretical MIC was calculated by assignment “y” is “95”.

2.4 Fecal microbiota transplantation

Fecal microbiota transplantation (FMT) experiment was conducted based on a previous study [18]. Briefly, twelve C57BL/6J mice (SPF class, male, 4 weeks of age) were fed a LFD for 8 weeks and used as donor mice of normal gut microbiota. Meanwhile, another twelve C57BL/6J mice (SPF class, male, 4 weeks of age) were accommodated for 3 days, and then had free access to LFD and antibiotic cocktails containing vancomycin (0.5 g/L), neomycin sulfate (1 g/L), metronidazole (1 g/L) and ampicillin (1 g/L) in water for consecutive 3 weeks. Next the mice were randomly divided into 2 groups (n = 6), and administered with the fecal microbiota of donor mice or *E. faecalis* (verify information was shown in Supplemental Fig. 2), respectively. Feces from donor mice were collected sterilely every day and resuspended in PBS at 0.125 g/mL, followed by low-speed centrifugation (800 × g) for 10 min. A volume of 0.15 mL of the supernatant or the third generation of BHI-cultured *E. faecalis* (1.75×10^{11} CFU/mL) was administered to mice by oral gavage once a day for consecutive 7 days, and then fed with LFD for another 11 weeks.

2.5 Histomorphological analysis of fat and liver tissue

Intraperitoneal fat and liver tissue samples were fixed in 4% paraformaldehyde or fat fixative (G1119, Servicebio Technology Co., Ltd. Wuhan, Hubei, China), respectively for at least 24 h until slice production. First, 4 μm serial sections prepared by a microtome (G-P1, Servicebio, Wuhan, China) were stained with hematoxylin and eosin (H&E) after paraffin embedded by using an automated tissue processor (G-L5/P5, Servicebio, Wuhan, China). Second, liver was dehydrated in 15% sucrose solutions and then sectioned into 8–10 μm by cryostat for staining with oil red. The histological profiles were scanned by using Panoramic MIDI (3DHISTECH Ltd., Budapest, Hungary) and further analyzed by CaseViewer (Version 2.0, 3DHISTECH Ltd., Budapest, Hungary).

2.6 Analysis of biochemical indicators and cytokines in serum

Blood sera were obtained by centrifuging at 1500 × g for 10 min after coagulation at room temperature for 30 min. Levels of aspartate aminotransferase (AST), alanine transaminase (ALT), total cholesterol (TC), total glyceride (TG), high density lipoprotein cholesterol (HDL-c) and low density lipoprotein cholesterol (LDL-c) in serum were detected in terms of manufacturers' instructions with a MINDRAY BS-200 chemistry analyzer (MINDRAY Medical International Co., Shenzhen, China). The level of glucose in serum was measured with a glucose assay kit (Shanghai Rongsheng Biotech Co., Ltd., Shanghai, China), and the level of insulin in serum was detected by a Mouse INS ELISA Kit (E-EL-M1382c, Elabscience Biotechnology Co., Ltd., Wuhan, Hubei, China). Insulin resistance was reflected by HOMA-IR (homeostasis model assessment-estimated insulin resistance) according to the following formula [19]:

$$\text{HOMA-IR} = \text{fasting insulinemia (mU/L)} \times \text{fasting glycemia (mg/dL)} / 405$$

Levels of inflammatory cytokines in serum including interleukin (IL)-1β, IL-2, IL-6, tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1) were measured with respective ELISA kit (Thermo Fisher Scientific Inc., Rockford, IL, USA), and TNF were measured by flow cytometry (BD Accuri C6, San Jose,

CA, USA) with a BD™ CBA Mouse Inflammation Kit (ID552364, BD Biosciences, San Jose, USA) according to the manufacturer's manual.

2.7 Measurement of indicators of oxidative stress in liver

Liver samples of mice were immediately collected into sterile tubes after weighing and frozen in liquid nitrogen before store at -80 °C until use. The level of malondialdehyde (MDA) was measured with an assay kit (Nanjing Jiancheng Bioengineering Research Institute Co., Ltd., Nanjing, Jiangsu, China) according to the manufacturer's manual.

2.8 Analysis of gut microbiota

Feces were collected at the first day and final day of experiment, and recorded as Feces^{0W} and Feces^{12W}. Cecal contents of mice were collected after sacrifice and recorded as Cecal contents^{12W}. Total DNA was extracted by using a Stool DNA Isolation Kit (Tiangen Biotech Co., Ltd., Beijing, China). DNA quality was detected and controlled by Nanodrop (Thermo Fisher Scientific Inc., Rockford, IL, USA), and then 30 ng DNA was used for PCR amplification. The V4 hypervariable region of the bacterial 16S rRNA gene was amplified by PCR, where the forward primer was 515a: 5'-GTGCCAGCMGCCGCGGTAA-3' and the reverse primer was 806: 5'-GGACTACHVGGGTWTCTAAT-3'. For each cecal sample, a 10-digit barcode sequence was added to the 5' end of the forward and reverse primers (provided by Allwegene Technology Inc., Beijing, China), and each sample was carried out by three same replications to mitigate reaction-level PCR biases. The volume of PCR reaction was 25 µL, containing 12.5 µL of 2xTaq PCR MasterMix, 1 µL of forward and 1 µL of reverse primers at the concentration of 5 µM respectively, 3 µL BSA at the concentration of 2 ng/µL, 3 µL DNA samples (30 ng), and 4.5 µL double-distilled H₂O (ddH₂O). Cycling parameters were 95 °C for 5 min, followed by 25 cycles at 95 °C for 45 s, 50 °C for 50 s, and 72 °C for 45 s, and a final extension at 72 °C for 10 min. Detection parameters of agarose gel electrophoresis were referred as follows: concentration of gel with 1%, voltage with 170 V and electrophoretic time with 30 min. Three PCR products from the same sample were mixed in equidensity ratios and purified with a GeneJET Gel Extraction Kit (Thermo Fisher Scientific Inc., Rockford, IL, USA), quantified using real-time PCR, and sequenced at Allwegene Technology Inc. (Beijing, China).

2.9 Lipidomics

Liver samples (50 mg) was homogenized with 1 mL lysis buffer including methanol, methyl tert-butyl ether (MTBE) and internal standard mixture (MTBE: methanol = 3:1) for 2 min. The homogenate was then mixed with 500 µL of pure water for 1 min before centrifuging at 10,000 g for 10 min at 4 °C. The supernatant (500 µL) was concentrated and redissolved in 100 µL mobile phase B solution. The sample extracts were analyzed by using a LC-ESI-MS/MS system (UPLC, Shim-pack UFLC SHIMADZU CBM30A system; MS, QTRAP® 4500 System). The analytical condition was as follows, column, Thermo C30 (2.6 µm, 2.1 mm×100 mm); solvent A, acetonitrile/water (60:40 v/v), 0.04% acetic acid, 5 mmol/L ammonium formate; solvent B, acetonitrile/isopropanol (10:90 v/v), 0.04% acetic acid, 5 mmol/L ammonium formate; gradient program, A/B(80/20 at 0 min, 50/50 at 3 min, 35/65 at 5 min, 25/75 at 9 min, 10/90 at 15.5 min); flow rate, 0.35 mL/min; temperature, 45 °C; injection volume, 2 µL. The effluent was alternatively connected to an ESI-triple quadrupole-linear ion trap (QTRAP)-MS.

2.10 Statistical analysis

Results are expressed as means \pm SD. Significant differences between groups were determined using one-way analysis of variance (ANOVA) tests, followed by Fisher's least significant difference (LSD) and Duncan's Multiple Range test (SPSS21, IBM Corp., Armonk, NY, USA). Correlation of gut microbiota, serum indicators and hepatic metabolites was determined using Pearson's correlation analysis. A probability of $P < 0.05$ was considered significant.

3 Results

3.1 C3G, PCA and PGA showed protective effect against NAFLD in mice

As shown in Figure 1A, HFD induced a significant increase in the body weight (BW) of mice ($P < 0.05$ vs LFD group). Dietary supplementation of C3G, PCA or PGA, but not VA or FA, decreased the BW of HFD-fed mice. Histological analysis of liver showed that HFD led to diffuse bullous lipids accumulation around hepatic vein, which was attenuated by supplementation of C3G, PCA or PGA (Figure 1B). Correspondingly, C3G decreased serum ALT level (Figure 1C), while PCA and PGA reduced AST (Figure 1D) and ALT levels in HFD-fed mice ($P < 0.05$). Further analysis on the serum indicators revealed that HFD significantly increased the level of glucose (Figure 1E) and decreased the ratio of HDL-c to LDL-c ($P < 0.05$) (Figure 1F), but were recovered by supplementation of C3G, PCA or PGA.

Inflammatory cytokines were then measured in serum to evaluate the inflammation induced by HFD. As shown in Figure 1G-K, supplementation of C3G, PCA or PGA significantly decreased the overproduction of IL-1 β (G), IL-2 (H), IL-6 (I) and TNF- α (J) in HFD-fed mice, while PCA and PGA also decreased the level of MCP-1 (K) ($P < 0.05$). Supplementation of VA decreased serum levels of IL-1 β , IL-2 and IL-6, but showed no significant effect on TNF- α and MCP-1, while FA only decreased the level of IL-2.

3.2 PCA showed a dose-dependent effect against NAFLD by attenuating insulin resistance in mice

A dose-dependent experiment was further conducted to understand the protective effect of PCA on NAFLD. As shown in Figure 2, dietary supplementation of 0.025% to 0.1% PCA decreased the BW (A) of HFD-fed mice in a dose-dependent manner, which was consistent with the intraperitoneal fat (B) and liver weight (C). Particularly, PCA reduced the adipocyte size (D) in intraperitoneal fat deposition (B), and ameliorated diffuse bullous lipids accumulation in liver (E&F). As the marker of liver damage, PCA dose-dependently decreased levels of AST (G1) and ALT (G2) in serum, which were in accord with the level of MDA (H), a product of lipid peroxidation. Moreover, PCA recovered the HDL-c/LDL-c ratio (I) and decreased the production of TNF (J), which were consistent with the HOMA-IR (K), an index of insulin resistance. Lipidomics analysis of liver also showed that PCA significantly down-regulated the lipid metabolites in the pathways associated with insulin resistance (Supplemental Figure 1C).

3.3 PCA alleviated NAFLD potentially by down-regulating the relative abundance of *Enterococcus*

Gut microbiota has been considered as the key factor that affects development of NAFLD. Thus, gut microbiota was then been characterized by 16S rDNA gene sequencing. At the phylum level (Table 1), HFD

increased the relative abundance of Firmicutes both in feces and cecal contents ($P < 0.05$), but decreased the relative abundance of Bacteroidetes, which led to a significant increase in Firmicutes/Bacteroidetes ratio ($P < 0.05$). Supplementation of 0.1% PCA restored the ratio of Firmicutes to Bacteroidetes ($P < 0.05$). Further analysis at the genus level (Table 2) revealed that supplementation of PCA dose-dependently decreased the relative abundance of *Roseburia*, *Intestinibacter* and *Enterococcus*, which belong to Firmicutes.

Based on all data of each mouse, the correlation between cecal microbiota, serum indicators and hepatic lipid metabolites were analyzed by Pearson's correlation analysis. As shown in Figure 4A, 3 kinds of microbial genera that down-regulated by PCA were positively ($P < 0.05$) associated with the level of LDL-c (*Roseburia*, *Enterococcus*, *Intestinibacter*), ALT (*Roseburia*, *Enterococcus*, *Intestinibacter*), AST (*Roseburia*, *Enterococcus*, *Intestinibacter*), as well as endotoxin (*Roseburia*). Moreover, *Enterococcus* was positively ($P < 0.05$) associated with 12 kinds of significant hepatic lipid metabolites including Cer (m18:1/24:0), PS (18:0_0:0), PS (16:0/20:4), TG (14:0/20:0/20:1), TG (16:0/16:1/22:0), TG (14:0/20:1/20:1), TG (16:1/18:1/20:0), TG (14:0/20:1/22:2), TG (16:0/16:1/22:3), TG (12:0/22:1/22:3), TG (14:0/20:3/22:2), and TG (16:0/18:2/22:3). Meanwhile, *Roseburia* was positively ($P < 0.05$) associated with 5 kinds of significant hepatic lipid metabolites including Cer (m18:1/24:0), PS (18:0_0:0), TG (16:0/16:1/22:3), TG (14:0/20:3/22:2), and TG (16:0/18:2/22:3) (Figure 3B).

3.4 PCA showed a direct inhibitory effect against *E. faecalis* *in vitro*

Considering PCA dose-dependently decreased the relative abundance of *Enterococcus*, the bacteriostasis effect of PCA against *E. faecalis* was further evaluated *in vitro*. The results indicated that a concentration of 2 M PCA showed obvious bacteriostatic circle against *E. faecalis* (Supplemental Figure 2E). MIC experiment showed that a concentration of 22.727 mM PCA inhibited the growth of *E. faecalis* with the transmittance > 95% (Figure 4A). To access the accurate MIC of PCA against *E. faecalis*, a fitting curve between Concentration (x, mM) and Transmittance (y, %) was designed by converting "x" to "log₁₀(x)", and the theoretical MIC of PCA against *E. faecalis* is 12.457 mM based on the fitting curve: $\text{Log}_{10}(x_{\text{PCA}}) = 0.8647 - (\text{Log}_{10}(97.942/(y-2.458)-1))/5.348$ ($R^2 = 0.933$) (Figure 4B).

3.5 Transplantation of *E. faecalis* promoted NAFLD in mice

As *Enterococcus* showed significant correlation with 12 kinds of changed hepatic lipid metabolites in experiment 2. A FMT experiment was further designed to clarify the effect of *E. faecalis* in the pathogenesis of NAFLD (Figure 4C). As shown in Figure 4, transplantation of *E. faecalis* (*rE. faecalis*) increased the body weight (E) and intraperitoneal fat deposition (D&F) as compared with control group (*rLFD*), with a P value of 0.072 and 0.092, respectively. Meanwhile, *rE. Faecalis* significantly increase the liver weight (G) and induced obvious diffuse bullous lipids accumulation (H).

4. Discussion

Excessive energy intake^[20] and energy imbalance^[21] are considered as the primary factor causing overweight and obesity. Insulin resistance and abnormal lipids metabolism play an important role in the onset of NAFLD, typically characterized as high serum triglyceride, high LDL-c and low HDL-c levels^[22]. As

the first-hit to induce NAFLD, insulin resistance influenced by diets results in decrease of sugar utilization for tissues, together with the decreased sensitivity to insulin, which conversely aggravates the utilization of lipids for cells and a large amount of insulin accumulated as hyperinsulinemia [23]. Lipid accumulation in the liver is the main cause of cytotoxicity to hepatocytes, which induces hepatocyte injury and secondary inflammation/fibrosis during the disease [24]. In this study, HFD caused lipid metabolism disorder and insulin resistance (HOMA-IR) as reflected by the lower HDL-c/LDL-c ratio and higher levels of both glucose and insulin, accompanied by hepatic lipid accumulation and inflammation. Supplementation of C3G and its main phenolic metabolites PCA and PGA showed protective effect against NAFLD in mice, in particular, PCA dose-dependently ameliorated hepatic steatohepatitis and inflammation by attenuating insulin resistance, as reflected by lower levels of AST, ALT, MDA, TNF and HOMA-IR index, but higher HDL-c/LDL-c ratio.

Increased Firmicutes/Bacteroidetes ratio was reported to have a close correlation with obesity [25], and our results indicated that the Firmicutes/Bacteroidetes ratio was increased by HFD but decreased by PCA. At the genus level, PCA decreased the relative abundance of *Roseburia*, *Intestinibacter* and *Enterococcus* that belonging to Firmicutes, and the correlation analysis revealed that *Roseburia*, *Intestinibacter* and *Enterococcus* had a positive correlation with serum indicators of hepatic damage (AST & ALT). *Intestinibacter* was reported to be involved in mucus degradation and metabolic disorder in type-2 diabetes [26], and *Enterococcus* was considered as one of the major causative agents of infection in HFD-induced NAFLD [27]. *Enterococcus* can secrete cytolysin to cause hepatocyte injury [5], and metabolize trimethyllysine into N,N,N-trimethyl-5-aminovaleric acid so that reducing oxidation of fatty acids [6]. *Roseburia* is one of the short-chain fatty acid (SCFA)-producing bacteria, and closely associated with metabolic syndrome [28,29], which suggesting that dysbiosis of gut microbiota may cause disorder of SCFA production in NAFLD [30]. Other studies have also indicated that NAFLD patients have higher SCFA levels along with SCFA-producing bacteria [3]. Therefore, PCA might decrease the relative abundance of harmful bacteria in eutrophic state, such as *Intestinibacter* and *Enterococcus*, to maintain microecological balance and energy balance, and decrease the relative abundances of specific SCFA-producing bacteria like *Roseburia* to reduce the production of SCFAs to suppress lipogenesis and gluconeogenesis [30].

The metabolism of hepatic neutral lipid, mainly triacylglycerol, is critical in the development of NAFLD [31]. In this study, lipidomics analysis revealed that HFD significantly up-regulated 120 kinds of lipids metabolites, while 41 kinds of lipids metabolites were down-regulated (Supplemental Figure 1B), which suggesting that HFD-induced disorder of lipid metabolism involved in both up-regulation and down-regulation of lipids. Most of the HFD up-regulated lipid metabolites belong to triacylglycerols (data are not shown), which can explain the lipid accumulation in liver. Most of the lipid metabolites down-regulated by HFD, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), which were reported to be correlated with the composition of intestinal microvillus membrane [32], and possess anti-inflammatory activity to protect intestinal epithelial cells [33-35]. Thus, HFD might potentially increase cell membrane permeability and weakened the barrier function to promote the translocation of bacteria from gut to liver [36]. On the other hand, most of the lipid metabolites down-regulated by PCA are belonging to triacylglycerols (Supplemental Table 2), and KEGG analysis indicated that all these triacylglycerols are involved in insulin resistance-related pathways. The correlation analysis between gut microbiota and lipid

metabolites indicated that *Roseburia* and *Enterococcus* are positively correlated with the production of Cer (m18:1(4E)/24:0), which has been proved to induce insulin resistance^[37] and promote the development of NAFLD^[38]. Our results indicated that PCA showed a direct inhibitory effect on *E. faecalis*, a representative strain of *Enterococcus*, and FMT results revealed that *E. faecalis* aggravate the progress of NAFLD. Therefore, the protective effect of PCA on NAFLD was largely dependent on the attenuated insulin resistance by down-regulating the relative abundance of *Enterococcus*.

5. Conclusion

Dietary supplementation of PCA, a bioactive phenolic metabolite of polyphenol C3G attenuated insulin resistance with reduced hepatic lipids accumulation, inflammation and damage in a NAFLD model induced by HFD. Microbial sequencing, lipidomics and correlation analysis indicated that the genera *Enterococcus* had a significant correlation with the changed lipid metabolites by PCA. *In vitro* bacteriostatic experiment showed that PCA can inhibit the growth of *E. faecalis*, and FMT revealed that *E. faecalis* had a promoting effect on NAFLD in mice. These results demonstrated the first time that PCA can ameliorate NAFLD by inhibiting *E. faecalis* in mice.

Declarations

Ethics approval and consent to participate

The experimental procedures were approved by the Hunan Agricultural University Institutional Animal Care and Use Committee (Permission No. 2018001).

Consent for publication

Not applicable

Availability of data and materials

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Experimental execution, J. T., Y. L., R. H. and Z. H.; Writing—original draft preparation, J.T. and S. W.; Writing—review and editing, S.W., D.-X.H., J. H. and H. Z.; supervision, S.W. and J. H.

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Tables

Table 1 The effect of PCA on relative abundance of microbes at the phylum level.

Groups	Firmicutes (%)			Bacteroidetes (%)		
	Feces ^{0w}	Feces ^{12w}	Cecal contents ^{12w}	Feces ^{0w}	Feces ^{12w}	Cecal contents ^{12w}
LFD	36.37±7.07	69.44±5.00 ^a	68.92±10.39 ^a	44.76±8.53 ^b	6.35±3.07 ^c	14.7±11.22 ^b
HFD	42.15±7.32	87.85±4.52 ^c	87.33±2.13 ^b	27.24±3.63 ^a	2.33±0.76 ^a	5.21±1.78 ^a
LPCA	45.06±9.48	79.53±8.76 ^{bc}	82.22±8.05 ^b	33.59±15.81 ^{ab}	3.22±0.89 ^{ab}	5.85±2.49 ^a
HPCA	52.2±16.27	75.89±8.97 ^{ab}	81.08±5.50 ^b	27.11±11.75 ^a	5.49±2.68 ^{bc}	9.43±2.59 ^{ab}
	Firmicutes/Bacteroidetes					
	Feces ^{0w}	Feces ^{12w}	Cecal contents ^{12w}			
LFD	0.84±0.23	9.76±1.64 ^a	7.68±6.14 ^a			
HFD	1.59±0.47	41.33±13.31 ^c	18.47±6.30 ^c			
LPCA	1.56±0.58	25.89±5.38 ^b	16.45±7.87 ^{bc}			
HPCA	2.41±1.64	17.05±9.10 ^a	9.21±2.96 ^{ab}			

Notes. Data represent as mean ± SD (n=6), and number in the same column with different letters differ significantly ($P < 0.05$). HFD, high-fat diet; HPCA, HFD+0.1% protocatechuic acid; LFD, low-fat diet; LPCA, HFD+0.025% protocatechuic acid.

Table 2 The effect of PCA on relative abundance of microbes at the genus level.

Groups	<i>Roseburia</i> (%)			<i>Intestinibacter</i> (%)		
	Feces ^{0w}	Feces ^{12w}	Cecal contents ^{12w}	Feces ^{0w}	Feces ^{12w}	Cecal contents ^{12w}
LFD	0.82±0.40	0.85±0.42 ^a	1.79±1.31 ^a	N/A	1.29±0.34 ^a	0.77±0.68 ^a
HFD	0.59±0.44	4.91±3.44 ^b	6.68±0.82 ^c	N/A	15.67±7.06 ^b	6.17±3.53 ^b
LPCA	0.62±0.36	2.48±1.49 ^a	5.82±2.06 ^{bc}	N/A	4.20±4.25 ^a	2.39±1.84 ^a
HPCA	1.25±1.05	1.16±0.51 ^a	4.17±1.75 ^b	N/A	3.33±1.59 ^a	2.88±2.28 ^a
	<i>Enterococcus</i> (%)					
	Feces ^{0w}	Feces ^{12w}	Cecal contents ^{12w}			
LFD	0.03±0.02	0.02±0.01 ^a	0.01±0.01 ^a			
HFD	0.05±0.05	0.22±0.11 ^b	0.12±0.12 ^b			
LPCA	0.05±0.08	0.04±0.03 ^a	0.03±0.01 ^a			
HPCA	0.17±0.19	0.01±0.01 ^a	0.02±0.01 ^a			

Notes. Data represent as mean ± SD (n=6), and number in the same column with different letters differ significantly ($P < 0.05$). HFD, high-fat diet; HPCA, HFD+0.1% protocatechuic acid; LFD, low-fat diet; LPCA, HFD+0.025% protocatechuic acid; N/A, not applicable.

Figures

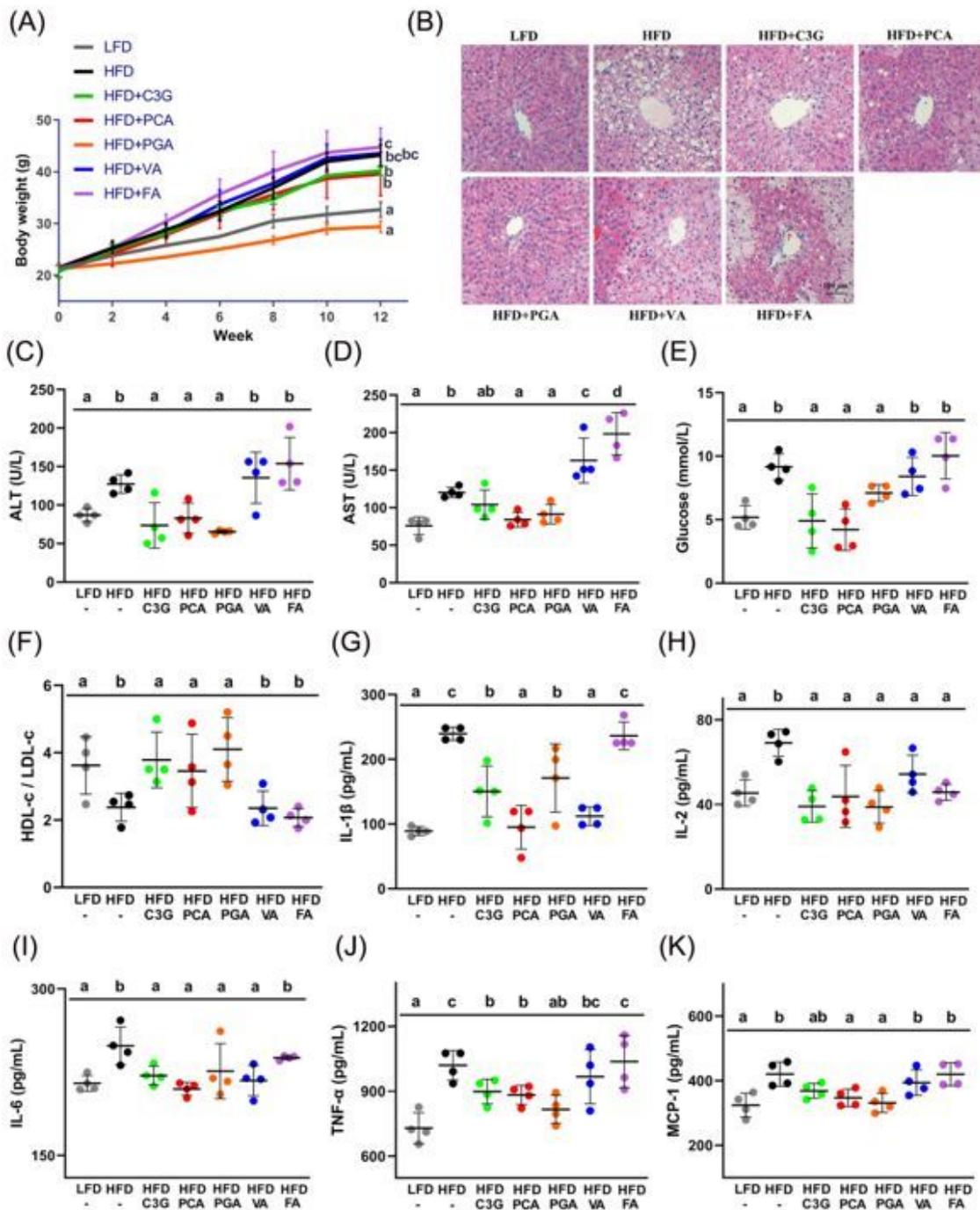


Figure 1

Effects of C3G, PCA, PGA, VA and FA on NAFLD. (A) Growth curves for body weight. (B) Representative sections of liver by hematoxylin–eosin (H&E) staining (original magnification $\times 100$). Levels of (C) ALT, (D) AST, (E) glucose and (F) HDL-c/LDL-c ratio in serum were measured using an automatic biochemical analyzer. Cytokines including (G) IL-1 β , (H) IL-2, (I) IL-6, (J) TNF- α and (K) MCP-1 in serum were measured with their respective ELISA kit. Data represent as mean \pm SD (n=4), and bars with different letters differ significantly ($P < 0.05$). ALT, alanine aminotransferase; AST, aspartate aminotransferase; C3G, cyanidin-3-glucoside; FA, ferulic acid; HDL-c, high density lipoprotein cholesterol; HFD, high-fat diet; IL, interleukin; LDL-c,

low density lipoprotein cholesterol; LFD, low-fat diet; MCP-1, monocyte chemoattractant protein-1; PCA, protocatechuic acid; PGA, phloroglucinaldehyde; TNF- α , tumor necrosis factor- α ; VA, vanillic acid.

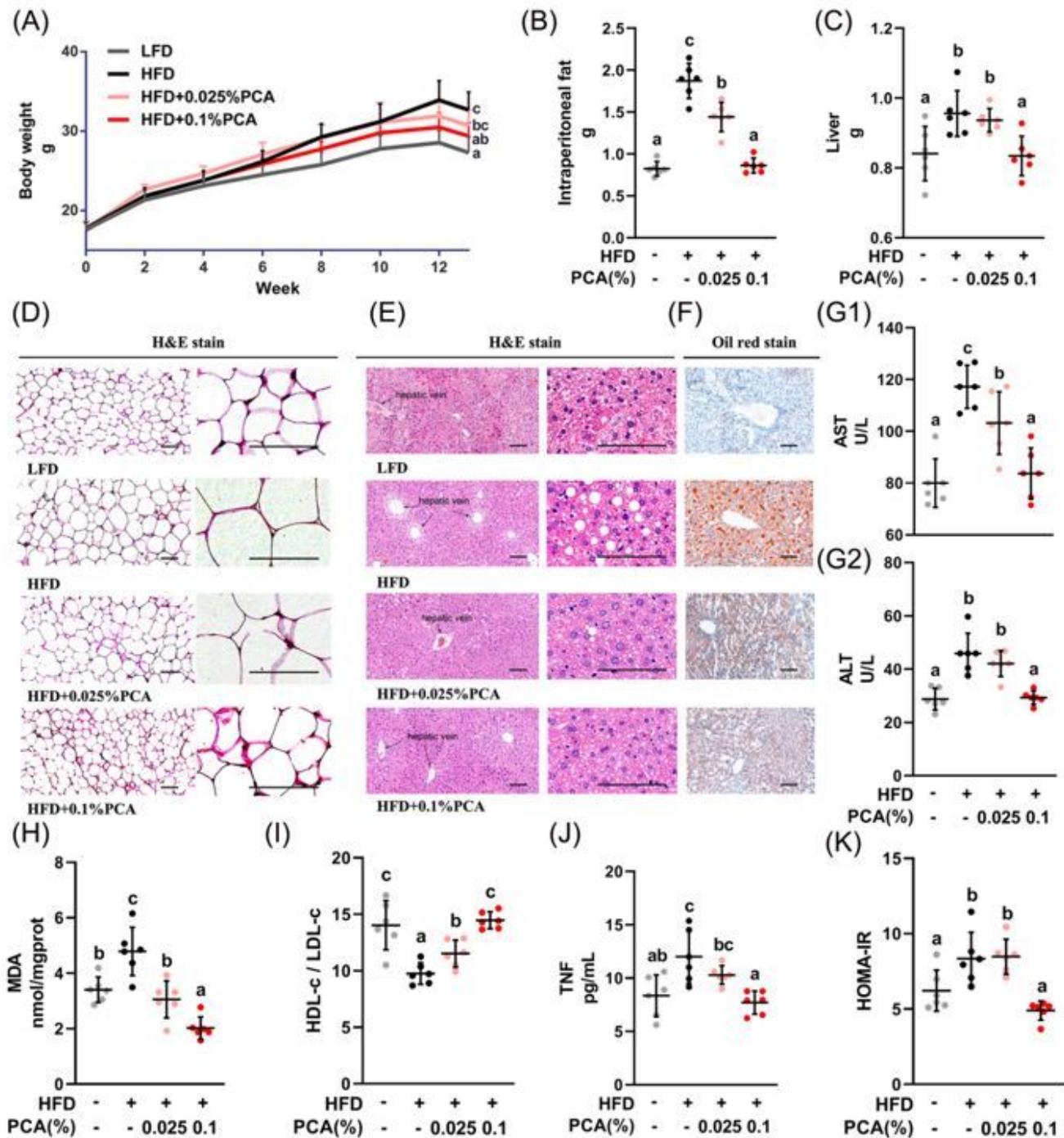


Figure 2

Dose-dependent effect of PCA against NAFLD in HFD-fed mice. (A) Growth curves for body weight. (B) Intraperitoneal fat weight. (C) Liver weight. (D) Representative sections of intraperitoneal fat by hematoxylin–eosin (H&E) staining (original magnification $\times 100$ (left) and $\times 400$ (right)). (E) Representative sections of liver by hematoxylin–eosin (H&E) staining (original magnification $\times 100$ (left) and $\times 400$ (right)). (F) Representative

Correlation of gut microbiota, serum indicators and hepatic lipid metabolites. (A) Correlation of gut microbiota and serum indicators. (B) Correlation of gut microbiota and hepatic lipid metabolites down-regulated by PCA. The intensity of the colors represented the degree of association (red, positive correlation; blue, negative correlation). Significant correlations were marked by *P < 0.05, **P < 0.01. ALT, alanine aminotransferase; AST, aspartate aminotransferase; Cerm, ceramide; Glu, glucose; HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; PS, phosphatidylserine; TG, triglyceride; g_ represents microbial genus.

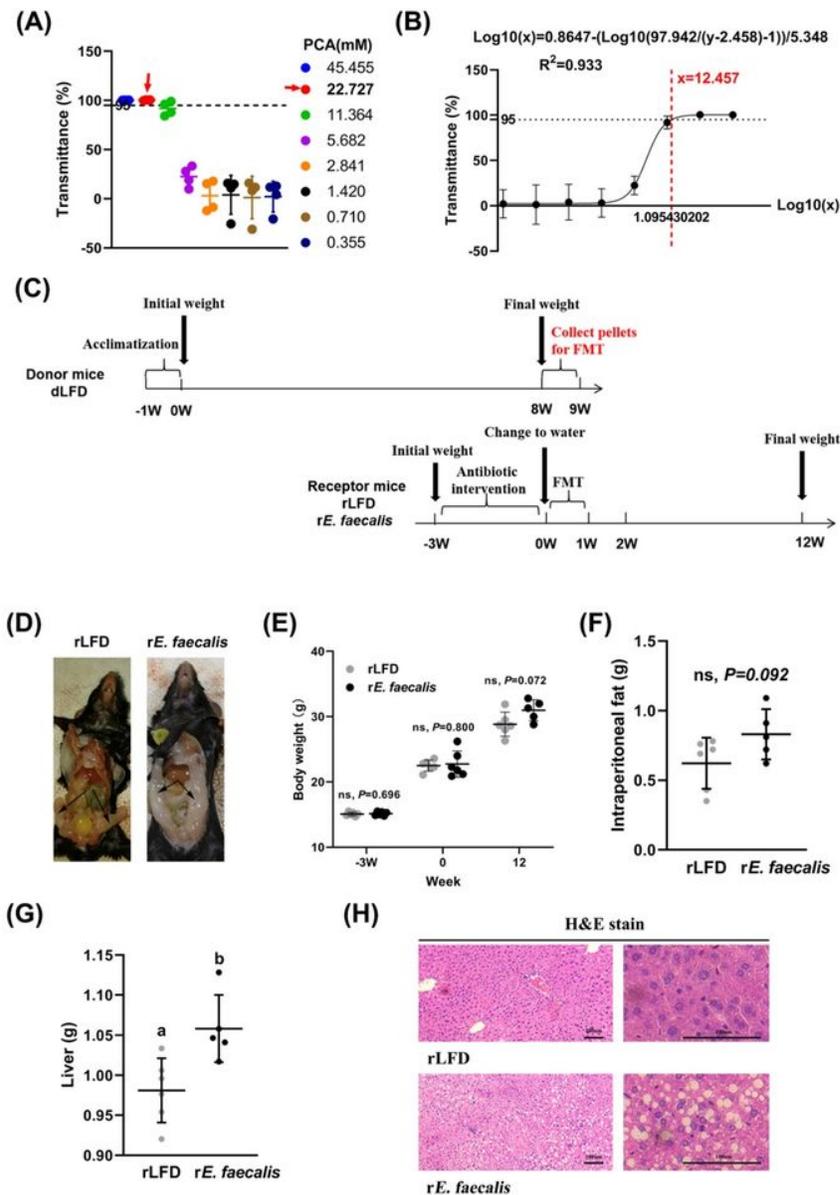


Figure 4

Bacteriostatic test of PCA against *E. faecalis* in vitro and promotion of *E. faecalis* for NAFLD in LFD-fed mice. (A) Results of transmittance in MIC experiment. (B) Fitting curve between Concentration (x, mM) and Transmittance (y, %). (C) Experimental design of FMT. (D) Anatomical drawing of rLFD and rE. faecalis. (E) Body weight. (F) Intraperitoneal fat weight. (G) Liver weight. (H) Representative sections of liver by hematoxylin–eosin (H&E) staining (original magnification $\times 100$ (left) and $\times 400$ (right)). Data represent as mean \pm SD. Bars with different letters differ significantly ($P < 0.05$). BHI, brain heart infusion; dLFD, donor mice of LFD; *E. faecalis*, *Enterococcus faecalis*; FMT, fecal microbiota transplantation; LFD, low-fat diet; MIC, minimum inhibitory concentration; PCA, protocatechuic acid; rE. faecalis, receptor mice of *E. faecalis*; rLFD, receptor mice of LFD.

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