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Supplementary Materials for

Citrus polymethoxyflavones attenuate metabolic syndrome by regulating gut microbiome and amino acid metabolism

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

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Supplementary Materials and Methods

Investigation of standard production process of Citrus PMFE. The effects of extraction and purification on the yield of PMFs were investigated and optimized by orthogonal tests for a standard production process of citrus PMFE. Firstly, 50 g of *Citrus reticulata* 'Chachi' peel sample was weighed into a 1000-mL round flask using heat reflux extraction (HRE) under different extraction conditions. The HRE conditions tested in our study include immersion time, extraction solvent, granule size, solid/solvent ratio, extraction time and times. Significant factors (independent variables) were optimized using Orthogonal design assistant 2.0, and every factor included 3 levels from low to high, respectively. The purification process of PMFs by macroporous resin chromatography has been investigated including static adsorption and desorption properties of macroporous resins and dynamic adsorption and desorption tests.

Quantitative and qualitative analysis of the major components in citrus PMFE. A total of 56 PMF compounds were chemically characterized by high performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (HPLC-QTOF/MS) according to the method published before (Zeng *et al.*, 2017). Quantitative analysis of four major flavonoids (sinensetin, nobiletin, 3,5,6,7,8,3',4'-heptamethoxyflavone and tangeretin) in citrus PMFE was conducted by high performance liquid chromatography coupled with diode array detector (HPLC-DAD). Chromatographic separation was performed on an Agilent ZORBAX SB C18 (4.6×250 mm, 5 μm) with an on-line filter in front of the column. The mobile phase consists of 1‰ formic-water (A) and acetonitrile (B), with a gradient elution as follows: 0-3 min, 25-50% B; 3-7 min, 50-58% B; 7-11 min, 58-58% B, 11-15 min, 58-70% B. Sample injection was 2 μL. The flow rate was set at 1 mL/min and the column temperature was 30 °C. The wavelength was set at 330 nm.

Viability assay. Cell viability was detected by MTT assay. Briefly, HL-7702 cells were seeded at the density of 2.5×10^5 cells/well in a 96-well plate. Cells were treated with PMFE as indicated. After 18 h, MTT (5 mg/mL) was added and incubated for 4 h. The cytotoxicity of PMFE was determined by microplate reader (Multiskan FC).

Nile-Red Staining. 100 ng/mL stock solution of Nile-red was prepared in PBS. After fixation with 4% paraformaldehyde (PFA) in PBS, cells were washed twice with PBS and stained with 100 ng/mL Nile-red in the dark for 10 min at room temperature followed by three washes with PBS. The nile-red stained cells were analyzed with Leica DMIRB (Nikon, Japan).

Epididymal fat and liver weight measurements. Freshly isolated epididymal adipose tissues and liver were weighed after the sacrifice of mice.

Oral Glucose tolerance test (OGTT) and insulin tolerance test (ITT). Oral Glucose tolerance test (OGTT) was performed on mice fasted overnight with free access to water, and insulin tolerance test (ITT) was tested after 6 h fast. Mice were intragastrically (i.g.) gavaged with 2 g/kg glucose (Sigma) or intraperitoneally (i.p.) injected with 0.75 U/kg insulin (Sigma). Blood glucose was measured in tail vein blood at 0, 15, 30, 60, 90, 120 min after the glucose gavage and 0, 30, 60, 90, 120, 150 min after insulin injection. Area under the curve (AUC) was calculated to quantify the OGTT and ITT results.

Biochemical measurements. Serum total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C) levels, Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) were measured by Automatic Biochemistry Analyzer (Cobas 8000) according to the manufacturer's instructions.

Histological analysis of liver, adipose and intestines. Livers, intestines, epididymal adipose tissues and interscapular brown adipose tissues were fixed in 4% paraformaldehyde and embedded in paraffin wax. Paraffin sections (5 μm) were stained with haematoxylin and eosin. Frozen liver sections were stained with oil red O and counterstained with hematoxylin to

visualize the lipid droplets. Sections were examined under digital pathological section scanner (NanoZoomer 2.0 RS, Hamamatsu).

16S rDNA amplicon sequencing. The QIIME data analysis package was used for 16S rDNA data analysis. The forward and reverse reads were joined and assigned to samples based on barcode and truncated by cutting off the barcode and primer sequence. The effective sequences were used in the final analysis. Sequences were grouped into operational taxonomic units (OTUs) using the clustering program VSEARCH (1.9.6) against the Silva 119 database pre-clustered at 97% sequence identity. The Ribosomal Database Program (RDP) classifier was used to assign taxonomic category to all OTUs at confidence threshold of 0.8.

Sequences were rarefied prior to calculation of alpha and beta diversity statistics. Alpha diversity indexes were calculated in QIIME from rarefied samples using for diversity the Shannon index, for richness the Chao1 index. Beta diversity was calculated using weighted and unweighted UniFrac and PCoA.

Table S1. PCR primers for detection of 10 *Bacteroides* species. Related to Fig. 6

| Species	Primer	Sequence of forward and reverse primers (5' to 3')
Bacteroides caccae	BaCAC	GGGCATCAGTTTGTTTGCTT
		GAACGCATCCCCATCTCATA
Bacteroides coprophilus	BaCPP	GGGTTGTAAACTTCTTTTGTGC
		GCCTCAACCGTACTCAAGGT
Bacteroides dorei	BaDOR	GGAAACGGTTCAGCTAGCAATA
		AGTCTTGTCAGAGTCCTCAGCATC
Bacteroides finegoldii	BaFIN	CCGGATGGCATAGGATTGTC
		CGTAGGAGTTTGGACCGTGT
Bacteroides fragilis	BaFRA	TGATTCCGCATGGTTTCATT
		CGACCCATAGAGCCTTCATC
Bacteroides ovatus	BaOVA	CCGGATAGCATACGAACATC
		CGTAGGAGTTTGGACCGTGT
Bacteroides stercoris	BaSTE	AAAGCTTGCTTTGATGGATG
		ACATACAAAAAGCCACACGTC
Bacteroides thetaiotaomicron	BaTHE	ATCAGACCGCATGGTCTTAT
		CAACCCATAGGGCAGTCATC
Bacteroides uniformis	BaUNI	TACCCGATGGCATAGTTCTT
		GGACCGTGTCTCAGTTCCAA
Bacteroides vulgatus	BaVUL	GCAGATGAATTACGGTGAAAGC
		GTCAGAGTCCTCAGCGGAAC
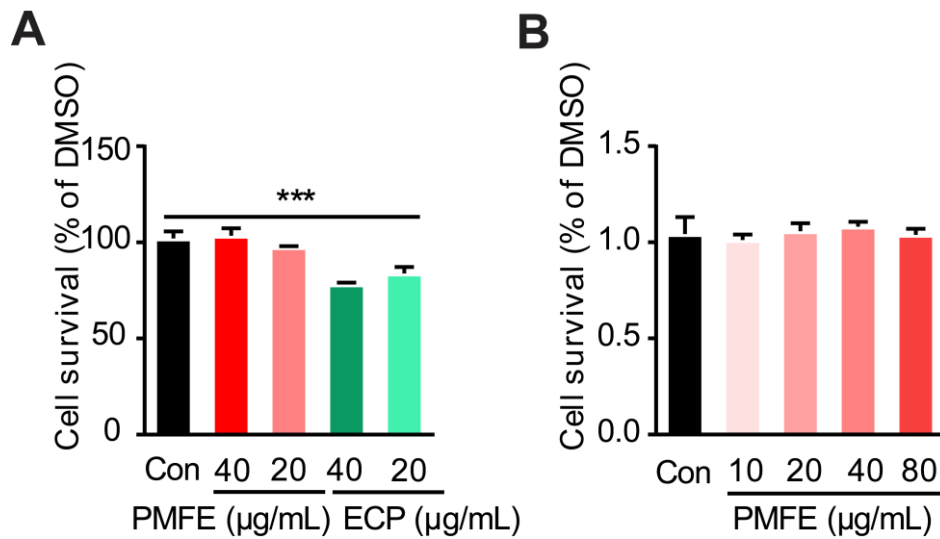
Fig. S1. Citrus PMFE shows negligible cytotoxicity in HL-7702 cells. Related to Fig. 1. (**A**) HL-7702 cells were treated with indicated concentrations of ECP and PMFE for 24 h. (**B**) HL-7702 cells were treated with indicated concentrations of PMFE for 24 h. Cell viability was measured by MTT assay. Statistically significant results were analyzed using one-way ANOVA with Turkey tests for multiple-group comparisons: (∗) $P < 0.05$, (∗∗) $P < 0.01$, and (∗∗∗) $P < 0.001$.
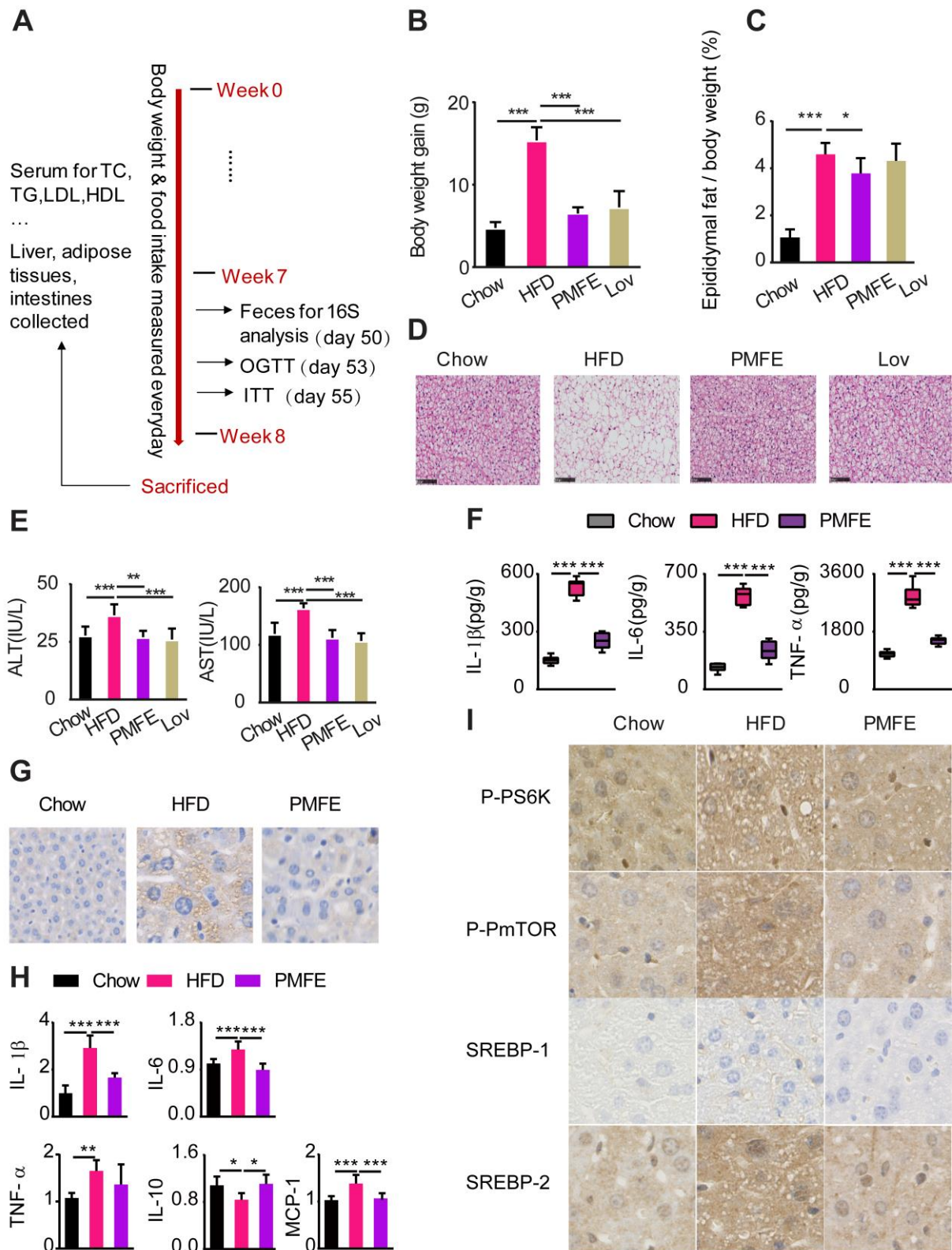
Fig. S2. Oral treatment of citrus PMFE prevents HFD-induced lipid deposition and inflammation in mice.

Related to Fig. 2. Mice were randomly divided into four groups (n=8). Chow-fed mice were treated daily with solvent (0.5% CMCNa) (Chow). HFD-fed mice were orally administered solvent (0.5% CMCNa) (HFD), 120 mg/kg/day PMFE (PMFE) or 30 mg/kg/day lovastatin (Lov). (**A**) The timeline of experimental design. (**B**) Body

weight gain. (**C**) Epididymal fat normalized by body weight. (**D**) Representative pictures of H&E-stained brown

adipose tissue. (**E**) Plasma ALT and AST. Data are presented as the mean value ± SD (n = 8) with bars. (**F**)

PMFE decreases the serum level of IL-1β, IL-6 and TNF-α. (**G**) PMFE decreased the macrophages in hepatic

tissues. Stained with anti-F4/80 antibody. (**H**) PMFE inhibited the mRNA expression of IL-1β, IL-6 TNF-α and

MCP-1 (Relative expression in comparison with Chow group). (**I**) PMFE inhibits the mTOR-P70S6K/SREBPs

pathway. Fixed liver tissues were stained with antibodies against SREBP-1, SREBP-2, P-PS6K and P-mTOR.

Statistically significant results were analyzed using one-way ANOVA with Turkey tests for multiple-group

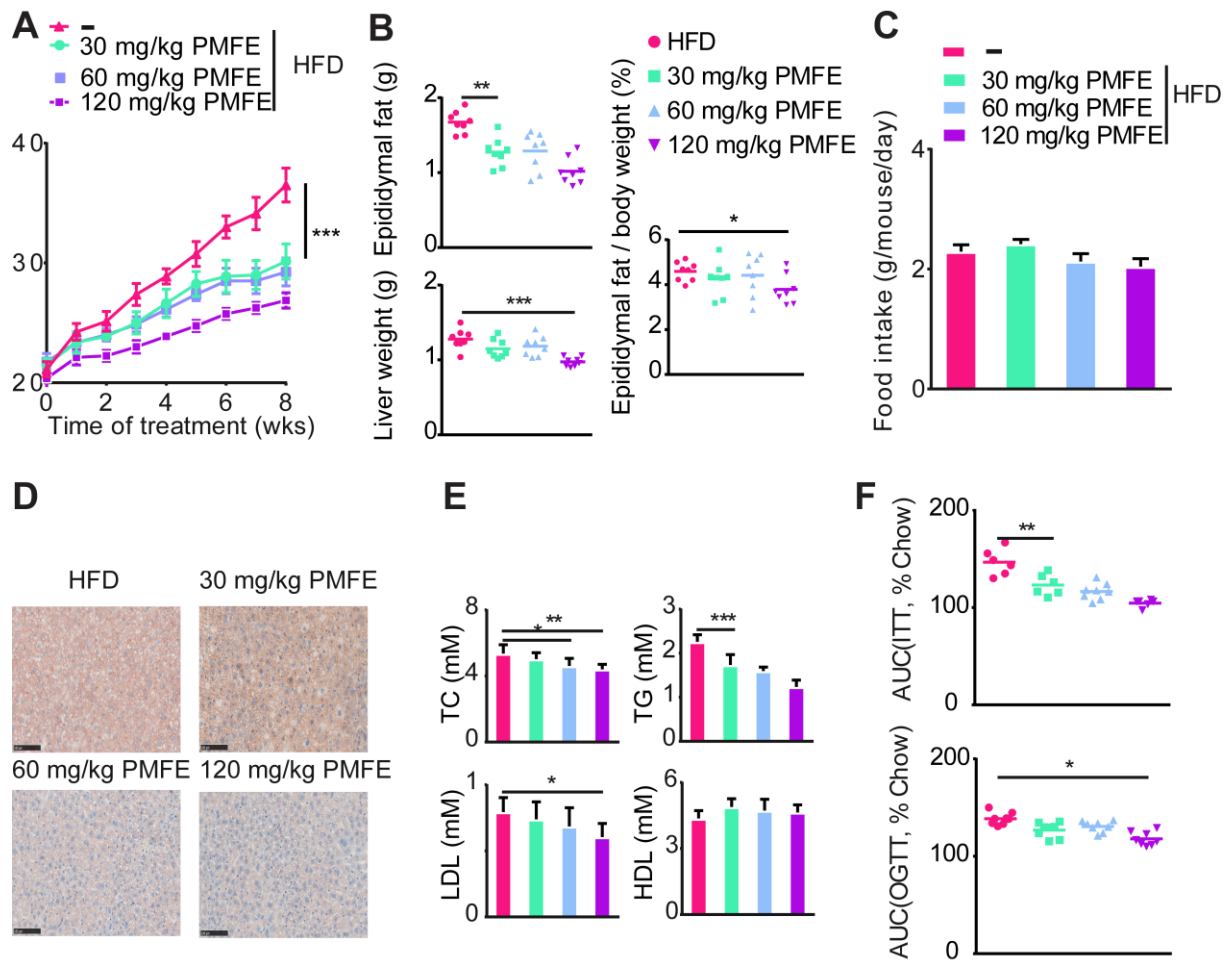comparisons: (∗) P < 0.05, (∗∗) P < 0.01, and (∗∗∗) P < 0.001. Scale bar 100 μm.

Fig. S3. Robust dose-dependent metabolic protection of citrus PMFE in HFD mice. Related to Fig. 2. Mice were randomly divided into the four groups (n=8). HFD-fed mice were treated daily with solvent (0.5% CMCNa), 30 mg/kg/day, 60 mg/kg/day or 120 mg/kg/day PMFE. (**A**) Body weight gain. (**B**) liver weight & epididymal fat (epididymal fat normalized to body weight). (**C**) The average daily food intake for the above four groups of mice. (**D**) Liver lipid content was assessed using oil red O staining. Scale bar 100 μm. (**E**) Total TC, TG, LDL, HDL levels. (**F**) AUC of ITT and OGTT are shown. Error bars are expressed as mean ± SD. Statistical significance was determined by one-way or two-way ANOVA with Turkey tests for multiple-group comparisons.
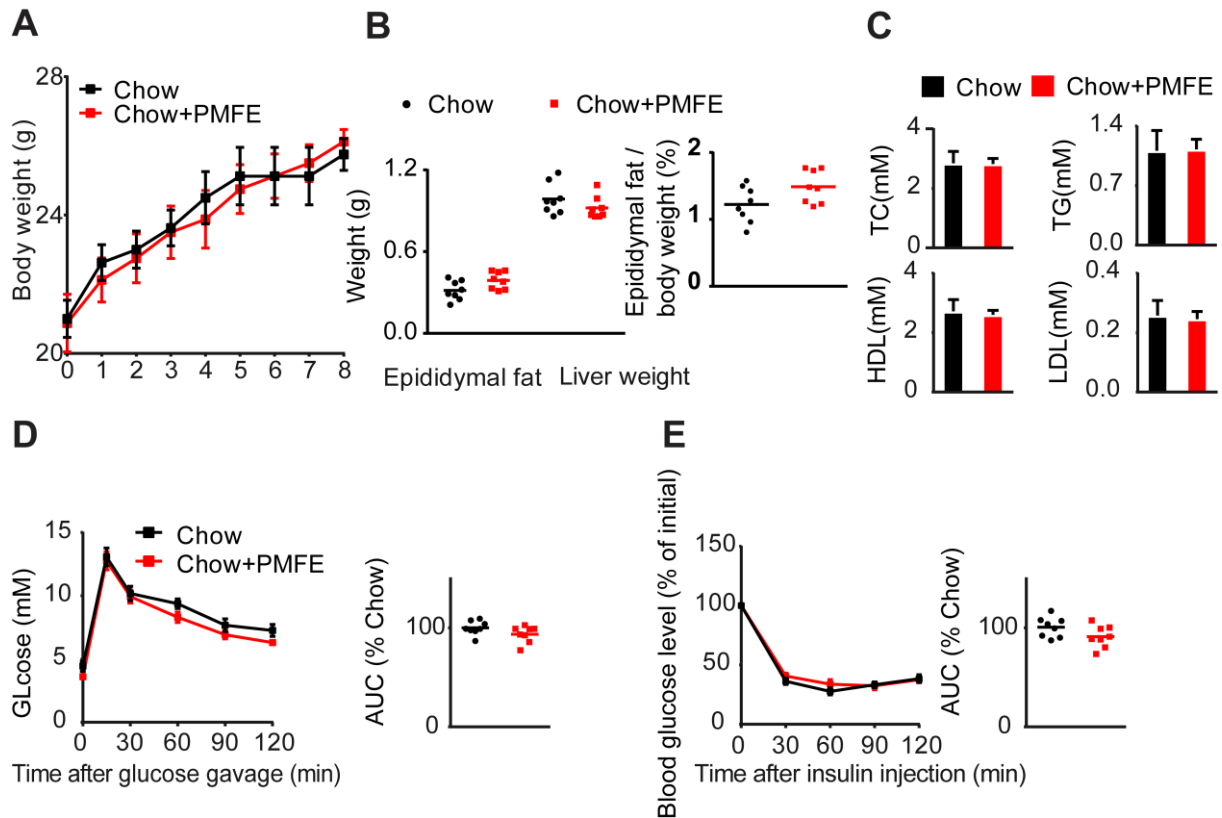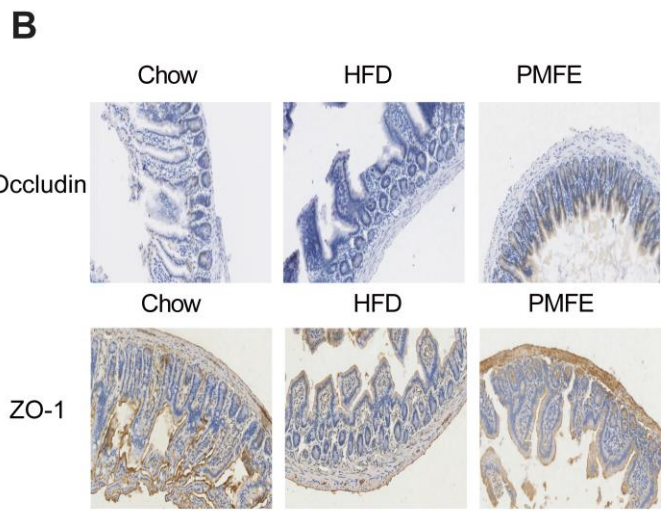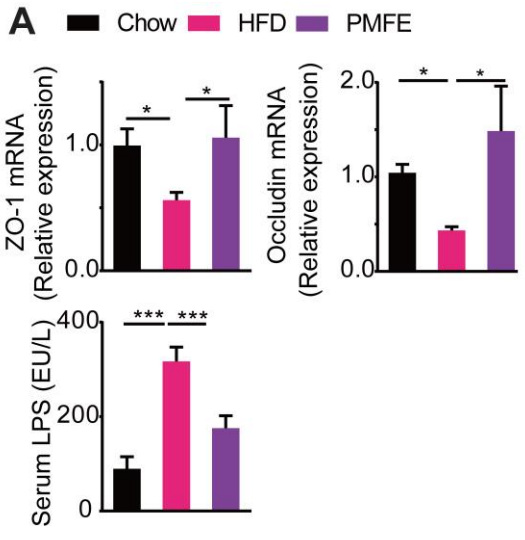
Fig. S4. Citrus PMFE does not produce any apparent effects in chow-fed mice. Related to Fig. 2. Mice were randomly divided into the two groups (n=8). Chow-fed mice were either treated daily with 0.5% CMC-Na (Chow) or 120 mg/kg/day PMFE (Chow + PMFE). (**A**) Body weight gain. (**B**) liver weight & epididymal fat (epididymal fat normalized to body weight). (**C**) Total TC, TG, LDL, HDL levels. (**D**) OGTT. (**E**) ITT. Error bars are expressed as mean ± SD. Statistical significance was determined by one-way or two-way ANOVA with Turkey tests for multiple-group comparisons.

A

Chow ■ HFD ■ PMFE ■

ZO-1 mRNA (Relative expression)

Occludin mRNA (Relative expression)

Serum LPS (EU/L)

B

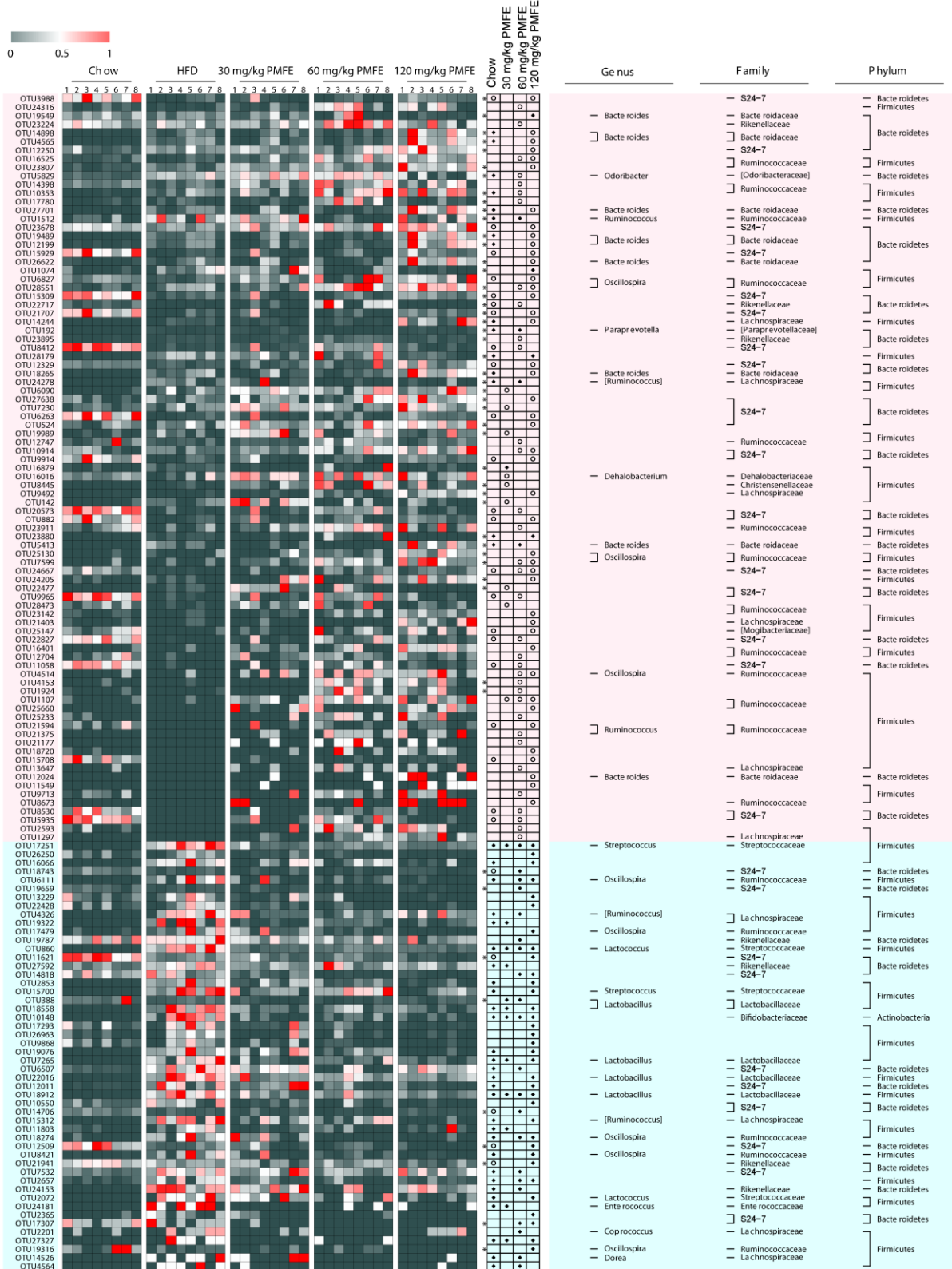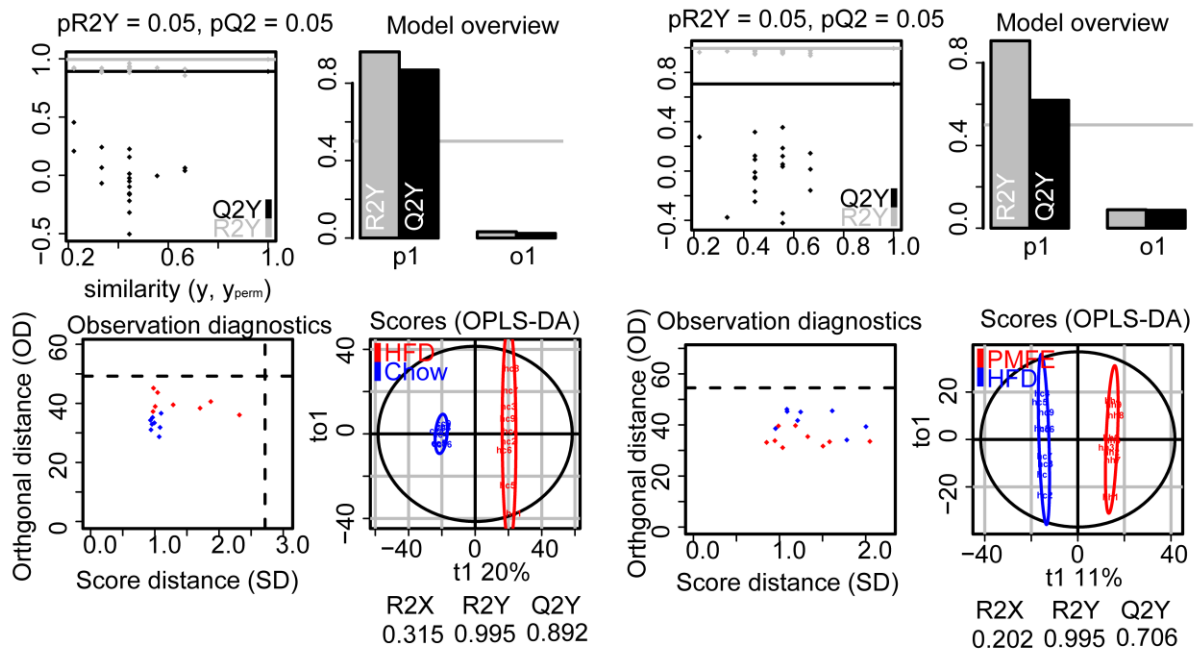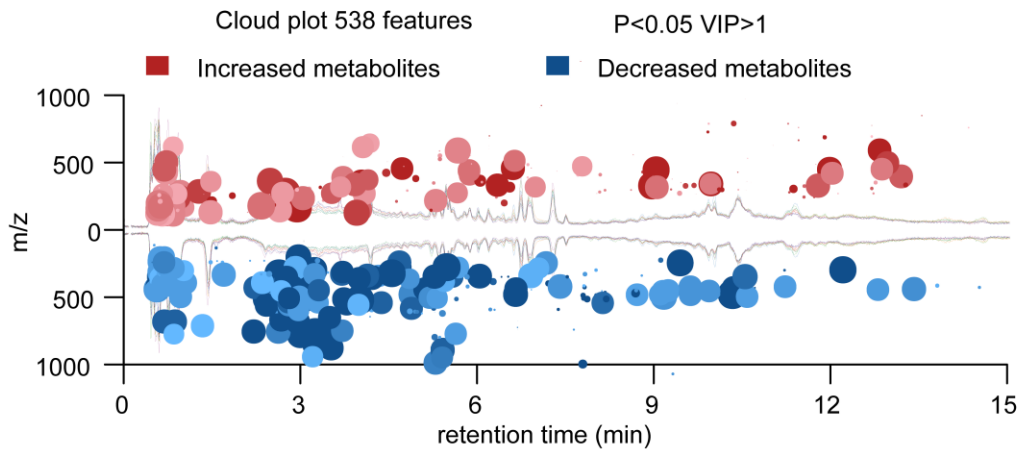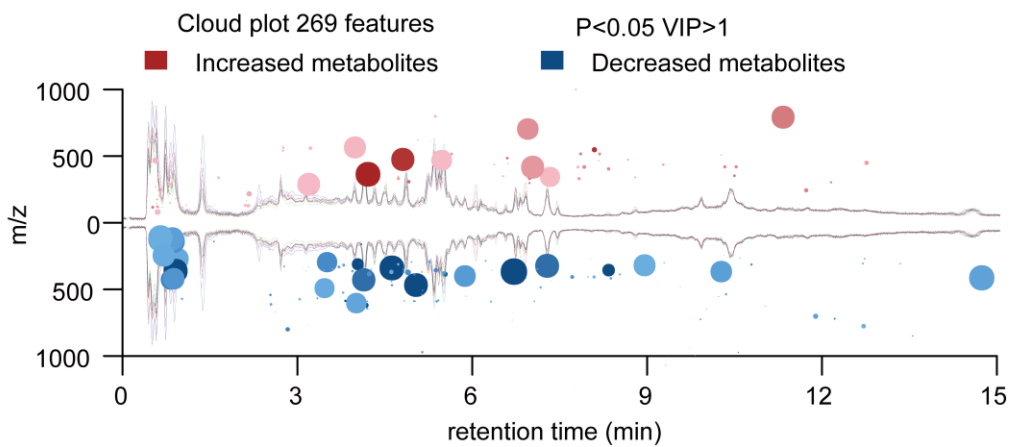|	Chow	HFD	PMFE
Occludin			
ZO-1			

Fig. S5. Citrus PMFE increases intestinal tight junction in HFD mice. Related to Fig. 3. Chow-fed mice were treated daily with solvent (0.5% CMCNa) (Chow). HFD-fed mice were orally administered solvent (0.5% CMCNa) (HFD) or 120 mg/kg/day (PMFE). (**A**) PMFE treatment reduces the mRNA expression of ZO-1,

occludin and lowers the serum level of LPS. (**B**) Representative immunoblots for ZO-1 and occludin in each group. (**C**) Heatmap of the 137 significantly altered OTUs altered upon HFD feeding compared with chow-fed mice. The color of the spots in the left panel represents the relative abundance of the OTU in each group. In the middle panel, white circles (○) represent OTUs less abundant in Chow and PMFE compared with HFD; Black diamonds (♦) represent OTUs more abundant in Chow and PMFE compared with HFD; Black star (*) represent OTUs in Chow changed by HFD was reversed by PMFE. The phylum, family and genus names of the OTUs are shown on the right panel.

A

pR2Y = 0.05, pQ2 = 0.05

Model overview

similarity (y, y_perm)

Observation diagnostics

Scores (OPLS-DA)

R2X   R2Y   Q2Y
0.315 0.995 0.892

pR2Y = 0.05, pQ2 = 0.05

Model overview

Observation diagnostics

Scores (OPLS-DA)

R2X   R2Y   Q2Y
0.202 0.995 0.706

B

Chow vs HFD feces

Cloud plot 538 features   P<0.05 VIP>1

■ Increased metabolites   ■ Decreased metabolites

HFD vs PMFE feces

Cloud plot 269 features   P<0.05 VIP>1

■ Increased metabolites   ■ Decreased metabolites

C

pR2Y = 0.05, pQ2 = 0.05

Model overview

Observation diagnostics

Scores (OPLS-DA)

R2X 0.373 R2Y 0.998 Q2Y 0.957

pR2Y = 0.05, pQ2 = 0.05

Model overview

Observation diagnostics

Scores (OPLS-DA)

R2X 0.265 R2Y 0.995 Q2Y 0.782

D

Chow vs HFD serum

Cloud plot 437 features   P<0.05 VIP>1

■ Increased metabolites   ■ Decreased metabolites

HFD vs PMFE serum

Cloud plot 280 features   P<0.05 VIP>1

■ Increased metabolites   ■ Decreased metabolites

Fig. S6. Citrus PMFE regulates host fecal and serum metabolome. Related to Fig. 4. Chow-fed mice were treated daily with solvent (0.5% CMCNa) vehicle (Chow). HFD-fed mice were orally administered solvent (0.5% CMCNa) vehicle (HFD) or 120 mg/kg/day (PMFE). (**A**) The OPLS-DA score plots for Chow vs HFD (feces) and HFD vs PMFE (feces) and corresponding permutation test. (**B**) The cloud plots for Chow vs HFD

(feces) and HFD vs PMFE (feces). (**C**) The OPLS-DA score plots for Chow vs HFD (serum) and HFD vs PMFE (serum) and corresponding permutation test. (**D**) The cloud plots for Chow vs HFD (serum) and HFD vs PMFE (serum). Chance permutation at 200 times was used for the OPLS-DA discrimination. Each bubble in the cloud plot corresponds to a metabolite feature, which includes visualization of the p-value, the directional fold change, the retention time, and the mass-to-charge ratio of features. The color of the bubble denotes directionality of fold change (up-regulated, red; down regulated blue) and the size of the bubble denotes the extent of the fold change (the larger the bubble, the larger the fold change). Statistical significance (p-value) is represented by the bubble's color intensity.
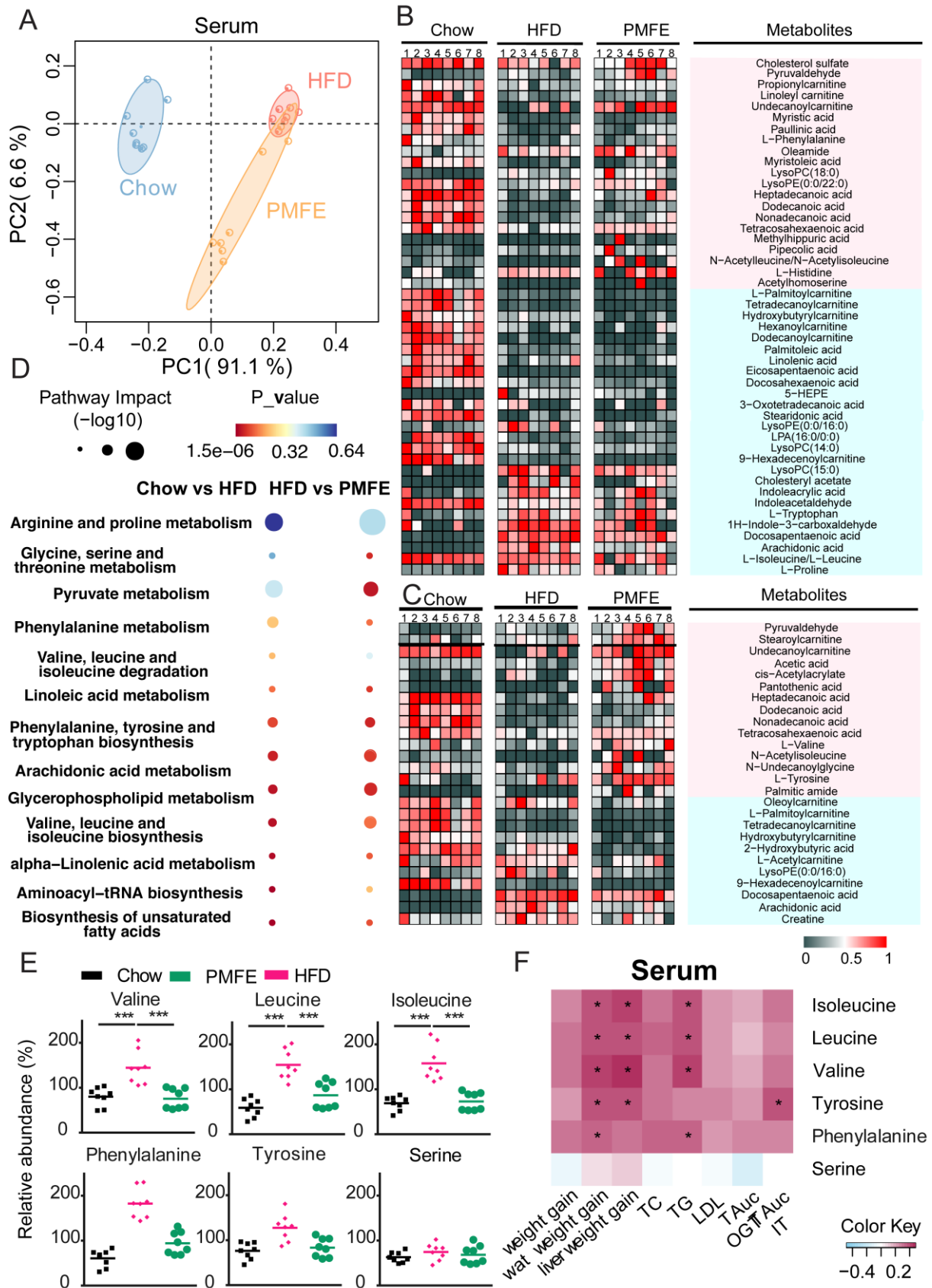
Fig. S7. Citrus PMFE alters MetS-associated BCAAs in HFD mice. Related to Fig. 4. Chow-fed mice were

treated daily with solvent (0.5% CMCNa) (Chow). HFD-fed mice were orally administered solvent (0.5%

CMCNa) (HFD) or 120 mg/kg/day (PMFE). (**A**) The Principal components analysis (PCA) score plots for discriminating the serum metabolome from normal group, control group and treatment group. (**B**) Heat maps of the differential serum metabolites that were altered by HFD feeding compared with chow-fed mice. (**C**) Heat maps of the differential serum metabolites that were altered by PMFE treatment compared with HFD-fed mice. (**D**) The disturbed metabolic pathways in the Chow vs HFD and HFD vs PMFE groups. (**E**) Comparison of circulating levels of valine, leucine, isoleucine, serine, and phenylalanine in serum by GC/MS in the indicated groups. (**F**) Heatmap analysis of the Pearson correlation of serum amino acids and metabolic syndrome related indexes. Red represents positive correlated and blue indicates negative correlated. Error bars are expressed as mean ± SD. Statistical significance was determined by one-way or two-way ANOVA with Turkey tests for multiple-group comparisons.
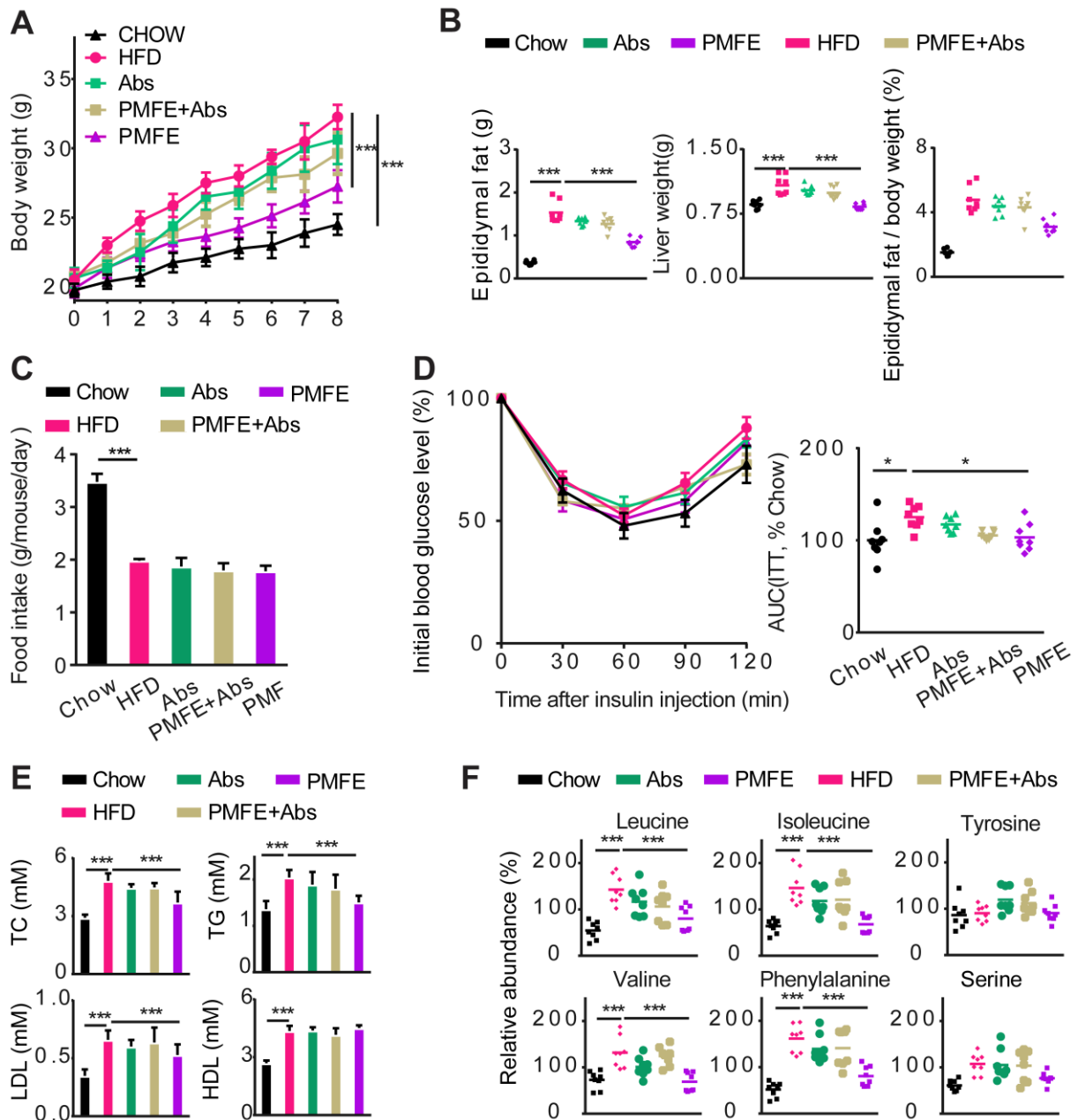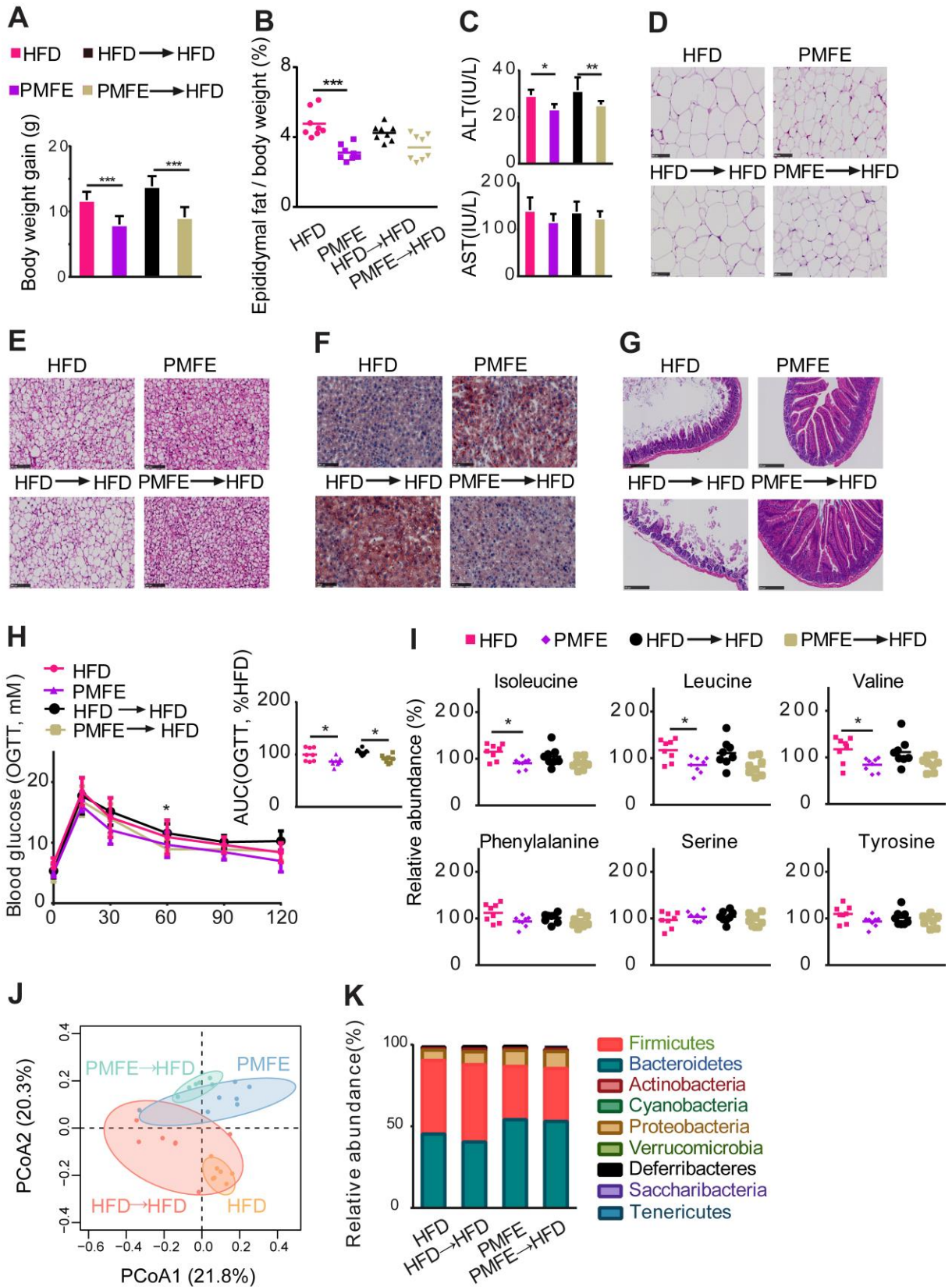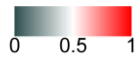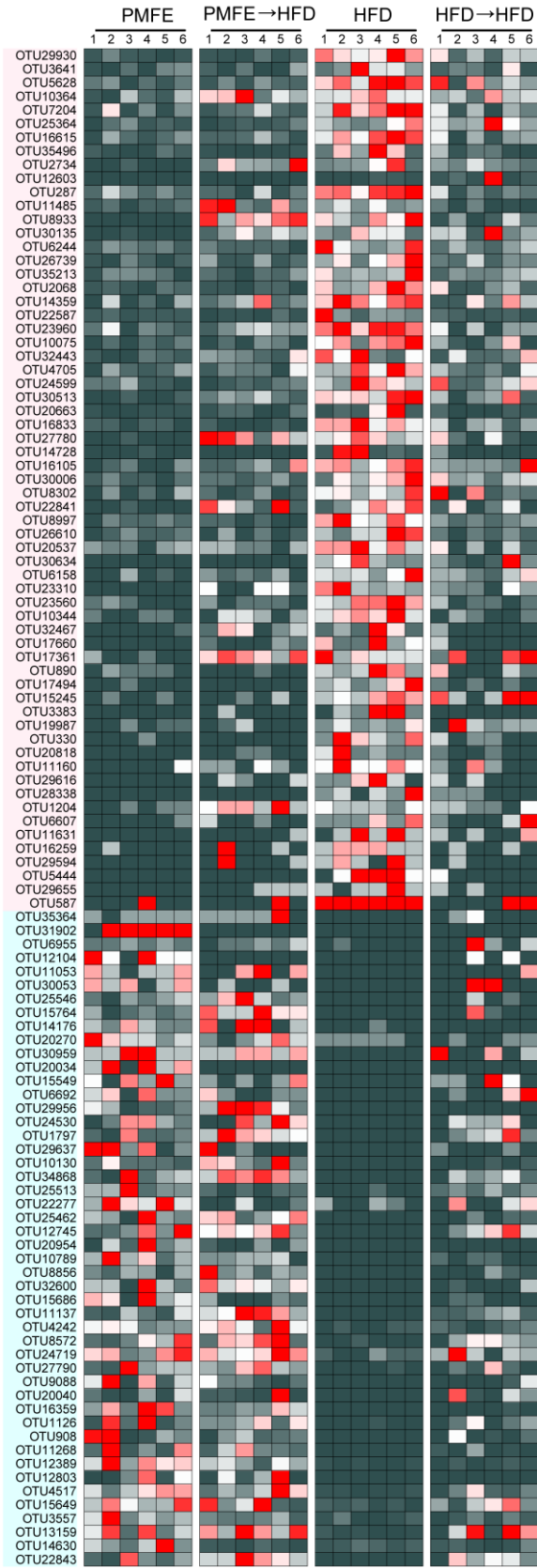
Fig. S8. Citrus PMFE attenuates MetS in HFD mice in a gut microbiota–dependent manner. Mice were randomly divided into five groups (n=8). Chow-fed mice were treated daily with solvent (0.5% CMCNa) (Chow). HFD-fed mice were orally administered solvent with Abs or without Abs (HFD) and 120 mg/kg/day PMFE in the presence (PMFE + Abs) or absence of Abs (PMFE). (**A**) Body weight of the above five groups of mice. (**B**) liver weight & epididymal fat (epididymal fat normalized to body weight) (**C**) The average daily food intake. (**D**) ITT. Right: AUC. (**E**) Total TC, TG, LDL and HDL levels in plasma. (**F**) The relative abundance of leucine, isoleucine, tyrosine, valine, phenylalanine and serine in feces by GC/MS in the indicated groups. Error bars are expressed as mean ± SD. Statistical significance was determined by one-way or two-way ANOVA with Turkey tests for multiple-group comparisons.

A

Body weight gain (g)

Legend: ■ HFD ■ HFD → HFD ■ PMFE ■ PMFE → HFD

***

B

Epididymal fat / body weight (%)

HFD PMFE HFD→HFD PMFE→HFD

***

C

ALT(IU/L)

*   **

AST(IU/L)

D

HFD   PMFE

HFD → HFD   PMFE → HFD

E

HFD   PMFE

HFD → HFD   PMFE → HFD

F

HFD   PMFE

HFD → HFD   PMFE → HFD

G

HFD   PMFE

HFD → HFD   PMFE → HFD

H

Blood glucose (OGTT, mM)

Legend: HFD, PMFE, HFD → HFD, PMFE → HFD

0 30 60 90 120

*

AUC(OGTT, %HFD)

*   *

I

Legend: ■ HFD ◆ PMFE ● HFD → HFD ■ PMFE → HFD

Relative abundance (%)

Isoleucine   Leucine   Valine

*   *   *

Phenylalanine   Serine   Tyrosine

J

PCoA2 (20.3%)

PMFE→HFD   PMFE

HFD→HFD   HFD

PCoA1 (21.8%)

K

Relative abundance(%)

HFD   HFD→HFD   PMFE   PMFE→HFD

Firmicutes
Bacteroidetes
Actinobacteria
Cyanobacteria
Proteobacteria
Verrucomicrobia
Deferribacteres
Saccharibacteria
Tenericutes

L

Fig. S9. Fecal transplantation of citrus PMFE exhibits metabolic protection in HFD mice. Related to Fig. 5. Mice were randomly divided into four groups (n=8). HFD-fed mice were orally administered solvent (0.5% CMCNa) (HFD) or 120 mg/kg/day PMFE (PMFE). Horizontal fecal transferred from solvent (0.5% CMCNa) -treated HFD mice are referred as HFD receivers (HFD→HFD). Horizontal fecal transferred from PMFE-treated mice are referred as PMFE receivers (PMFE→HFD). Mice were randomly divided into the four groups (n=8). HFD-fed mice were treated daily with solvent (0.5% CMCNa) or 120 mg/kg/day PMFE. (**A**) Body weight gain. (**B**) Epididymal fat normalized to body weight. (**C**) Plasma ALT and AST measured by the end of the week 8. (**D**) Representative pictures of H&E-stained white adipose tissue. (scale bars, 100 μm). (**E**) Representative pictures of H&E-stained brown adipose tissue. (scale bars, 100 μm). (**F**) Liver lipid content was assessed using oil red O staining (scale bar 100 μm). (**G**) Representative H&E pictures of intestine (scale bar, 250 μm). (**H**) OGTT. Right: AUC. (**I**) Relative abundance of valine, leucine, isoleucine, serine, and phenylalanine in serum by GC/MS in the indicated groups. (**J**) PCoA analysis of microbiota composition for PMFE, PMFE→HFD, HFD and HFD→HFD mice. (**K**) Bacterial taxonomic profiling in the phylum level of intestinal bacteria from different groups. (**L**) Heatmap showing the abundance of the 111 significantly altered OTUs responding to PMFE treatment in the PMFE, PMFE→HFD, HFD and HFD→HFD group. The color of the spots in the left panel represents the relative abundance of the OTU in each group. The phylum, family and genus names of the OTUs are shown on the right panel. Data are presented as the mean value ± SD (n = 8) with bars. Statistically significant results were analyzed using one-way ANOVA with Turkey tests for multiple-group comparisons: (∗) $P < 0.05$, (∗∗) $P < 0.01$, and (∗∗∗) $P < 0.001$.
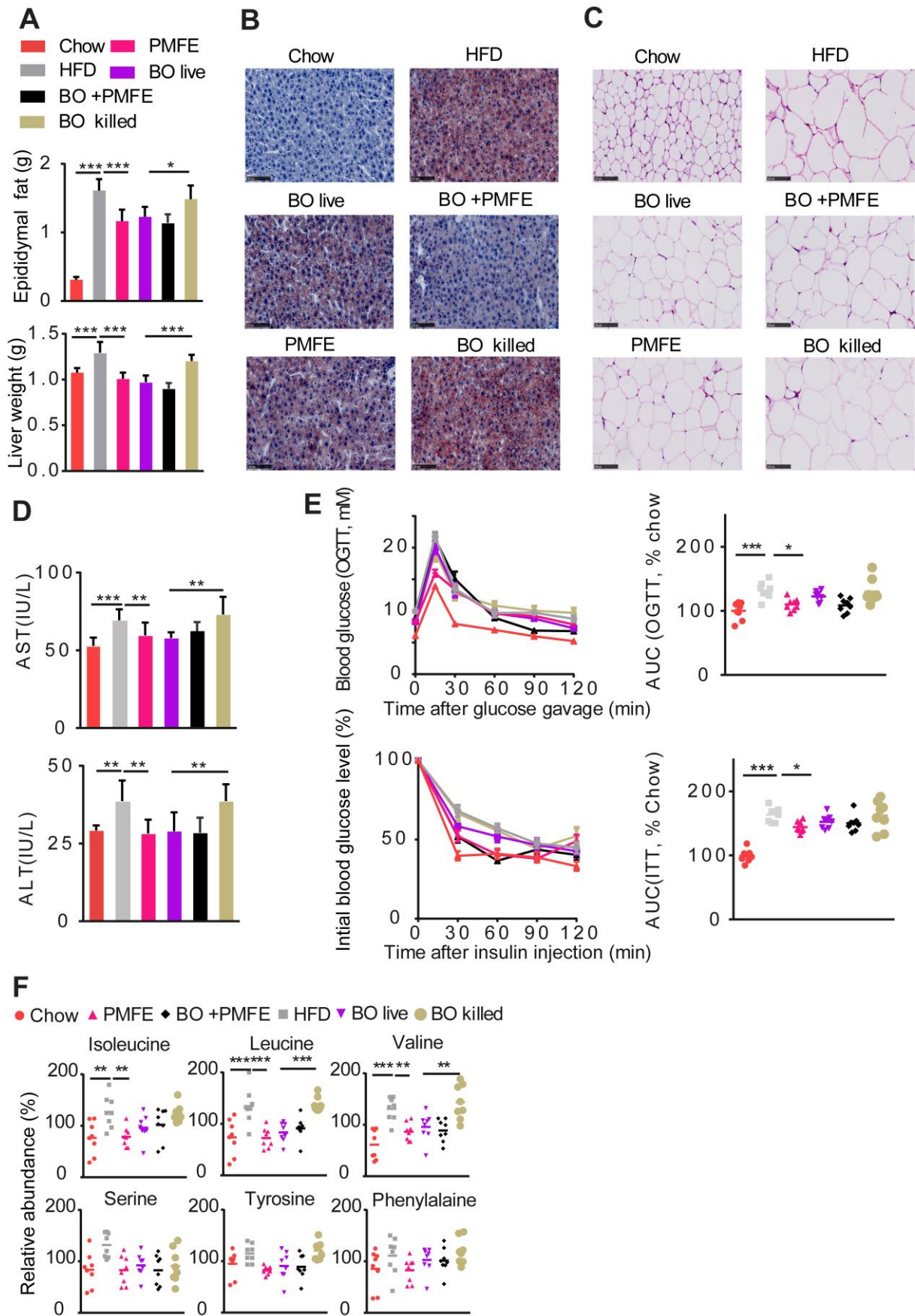
Fig. S10. Citrus PMFE–mediated enrichment of *B. ovatus* prevents metabolic syndrome in HFD mice.

Related to Fig. 6. Mice were randomly divided into four groups (n=8). Chow-fed mice were treated daily with solvent (0.5% CMCNa) (Chow). HFD-fed mice were orally administered with high dose PMFE (120 mg/kg/day, HFD+PMFE), *B. ovatus* (BO live), *B. ovatus* and high dose PMFE (BO+PMFE) or killed *B. ovatus* (BO killed). (**A**) liver weight & epididymal fat. (**B**) Liver lipid content was assessed using oil red O staining (scale bar 100 μm). (**C**) Representative pictures of H&E-stained white adipose tissue. (scale bars, 100 μm). (**D**) Plasma ALT and AST. (**E**) Glucose tolerance test and insulin tolerance test. (**F**) The relative abundance of valine, leucine, isoleucine, serine, and phenylalanine in serum by GC/MS in the indicated groups. Data are presented as the mean value ± SD (n = 8) with bars. Statistically significant results were analyzed using one-way ANOVA with Tukey tests for multiple-group comparisons: (∗) $P < 0.05$, (∗∗) $P < 0.01$, and (∗∗∗) $P < 0.001$.