Supplementary materials

Polyphenol- and caffeine-rich post-fermented Pu-er tea improves diet-induced metabolic syndrome by remodeling intestinal homeostasis in mice

Running title: Gut microbes link Pu-er tea and metabolic syndrome

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Supplementary tables and figures

Table S1 Important clones in DGGE profiles of mouse feces based on biplot analysis, with percent similarity to known sequences in GenBank

Band*	Length of 16S fragments (bp)	Aligned site in GenBank seq.	Similarity	Closest relatives in 16S rRNA sequence database	GeneBank Accession number
D-10	432	918 to 1346	416/429(97%)	*Eubacterium coprostanoligenes* strain HL	NR_104907.1
D-15	433	922 to 1353	432/432(100%)	*Romboutsia timonensis* strain DR1	NR_144740.1
D-19	419	939 to 1354	386/419(92%)	*Butyrivibrio crossotus* strain DSM 2876	NR_104735.1
D-21	454	930 to 1383	408/456(89%)	*Lachnoclostridium pacaense* strain Marseille-P3100	NR_147396.1
D-25	433	950 to 1382	403/433(93%)	*Eisenbergiella massiliensis strain AT11*	NR_041625.1
D-27	433	859 to 1291	403/433(93%)	[*Clostridium*] *bolteae* strain JCM 12243	NR_113410.1
D-28	431	777 to 1208	401/435(92%)	[*Clostridium*] *clostridioforme* strain ATCC 25537	NR_118128.1
D-32	432	930 to 1361	430/431(99%)	*Akkermansia muciniphila* strain ATCC BAA-835	NR_074436.1
D-49	434	932 to 1364	384/440(87%)	*Paenibacillus wenxiniae* strain 373	NR_145946.1
D-51-1	433	951 to 1383	384/433(89%)	[*Bacteroides*] *pectinophilus* strain N3	NR_121686.1
D-51-2	432	930 to 1361	432/432(100%)	*Akkermansia muciniphila* strain ATCC BAA-835	NR_074436.1
D-51-3	432	905 to 1330	384/431(89%)	*Muribaculum intestinale* strain YL27	NR_144616.1
G-13	432	918 to 1346	416/429(97%)	*Eubacterium coprostanoligenes* strain HL	NR_104907.1
G-19	431	966 to 1390	369/429(86%)	*Turicimonas muris* strain YL45	NR_144619.1
G-21	437	921 to 1353	433/433(100%)	*Romboutsia timonensis* strain DR1	NR_144740.1
G-26	431	965 to 1394	428/430(99%)	*Lactobacillus murinus* strain NBRC 14221	NR_112689.1
G-29	465	964 to 1424	427/466(92%)	*Lactobacillus murinus* strain NBRC 14221	NR_112689.1
G-33	431	942 to 1373	397/433(92%)	*Extibacter muris* strain 40cc-B-5824-ARE	NR_144610.1
G-38	437	950 to 1382	403/433(93%)	*Eisenbergiella massiliensis* strain AT11	NR_144731.1
G-41	418	996 to 1366	341/372(92%)	[*Ruminococcus*] *torques* strain VPI B2-51	NR_036777.1
G-58	434	932 to 1364	384/440(87%)	*Paenibacillus wenxiniae* strain 373	NR_145946.1
G-65	434	953 to 1383	381/431(88%)	[*Bacteroides*] *pectinophilus* strain N3	NR_121686.1
G-66	434	932 to 1361	430/430(100%)	*Akkermansia muciniphila* strain ATCC BAA-835	NR_074436.1
G-67	432	905 to 1331	393/432(91%)	*Muribaculum intestinale* strain YL27	NR_144616.1

*** All the bands were named according to the Figure 6A (band: D-) and 6D (band: G-).**

Table S2 Chemical characterization of post-fermented Pu-er tea (PE)

Category	Chemical components	Content (%)	Determination method
Polyphenols and their derivatives (Oxidized Tea Polyphenols)	Tea polyphenols*	22.80 ± 0.00	Ferric tartrate colorimetric method
	Catechins	1.01 ± 0.01	HPLC-UV
	Flavonoids	1.72 ± 0.05	HPLC-UV
	Gallic acid	1.35 ± 0.00	HPLC-UV
	Theabrownin	36.19 ± 0.01	Extraction-Spectrophotometry
	Theaflavin	5.20 ± 0.02	Extraction-Spectrophotometry
	Thearubigins	13.65 ± 0.01	Extraction-Spectrophotometry
Polysaccharides	Polysaccharides	14.16 ± 0.01	Anthrone-Sulfuric acid Colorimetric Method
Caffeine	Combined-Caffeine	2.59 ± 0.05	Low-pH precipitation method and HPLC-UV
	Free-Caffeine	7.37 ± 0.15	HPLC-UV
Protein and Amino acid	Protein	19.37 ± 0.02	Comassie brilliant blue colorimetric method
	Amino acid	4.83 ± 0.01	Automatic amino acid analyzer-External standard method
Others	Microelements (total)	63.39 mg/g	ICP-AES(GB/T 18932.11-2002)

*** For the complicated interaction between different components, it is difficult to purify the single substance from PE. Most of tea polyphenols (TP) in PE were oxidized, polymerized and condensed into theabrownin; Therefore, Ferric tartrate colorimetric method is not suit for the precise determination of TP in PE.**

Table S3 Chemical characterization of PE and TP

Catechins in PE		Microelements in PE		Chemical characterization of TP	
Catechins	**Content (%)**	**Elements**	**Content (μg/g)**	**Category**	**Content**
EGC	**0.34**	**K**	**50200**	**Purity (%)**	**≥ 98.0**
C	**0.16**	**P**	**9540**	**Catechin (%)**	**≥ 90.0**
EGCG	**0.90**	**Mg**	**2630**	**EGCG (%)**	**≥ 60.0**
EC	**0.36**	**Mn**	**726**	**Caffeine**	**< 0.5**
ECG	**0.60**	**Ca**	**263**	**Water content (%)**	**< 5.0**
		Fe	**37.01**	**Ash content (%)**	**< 0.2**
		Cu	**ND**	**Heavy metal (ppm)**	**< 10.0**
		Zn	**ND**	**Pesticide residue (ppm)**	**< 0.1**
		Na	**ND**		

EGC, (−)-epigallocatechin; C, (+)-catechin; EGCG, (−)-epigallocatechin-3-gallate; EC, (−)-epicatechin; ECG (−)-epicatechin-3-gallate.

Table S4 Primers sequences used for quantitative PCR analysis of gene expression

Primer	Forward sequence	Reverse sequence
RPL-19	GAAGGTCAAAGGGAATGTGTTCA	CCTTGTCTGCCTTCAGCTTGT
GAPDH	GTGTTCCTACCCCCAATGTGT	ATTGTCATACCAGGAAATGAGCTT
B2M	TTGTCTCACTGACCGGCCT	TATGTTCGGCTTCCCATTCTCC
TNF-α	AGACCCTCACACTCAGATCA	TCTTTGAGATCCATGCCGTTG
IL-1β	TCCATGAGCTTTGTACAAGGA	AGCCCATACTTTAGGAAGACA
IL-6	GTTCTCTGGGAAATCGTGGA	TGTACTCCAGGTAGCTA
MCP-1	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT
LPL	CATCGAGAGGATCCGAGTGAA	TGCTGAGTCCTTTCCCTTCTG
Fiaf	CAATGCCAAATTGCTCCAATT	TGGCCGTGGGCTCAGT
LOX-1	ACAAGATGAAGCCTGCGAAT	GCTGAGTAAGGTTCGCTTGG
G6pc	AGGAAGGATGGAGGAAGGAA	TGGAACCAGATGGGAAAGAG
Glut4	ACGACGGACACTCCATCTGTTG	GGAGACATAGCTCATGGCTGGAA
Cebpa	GAGCCGAGATAAAGCCAAACA	GCGCAGGCGGTCATTG
Fasn	TTCCAAGACGAAAATGATGC	AATTGTGGGATCAGGAGAGC
Acc1	TGTTGAGACGCTGGTTTGTAGAA	GGTCCTTATTATTGTCCCAGACGTA
Cpt1a	AGACCGTGAGGAACTCAAACCTAT	TGAAGAGTCGCTCCCACT
Acox1	CTATGGGATCAGCCAGAAAGG	AGTCAAAGGCATCCACCAAAG
Pgc1a	AGCCGTGACCACTGACAACGAG	GCTGCATGGTTCTGAGTGCTAAG
Ppara	CAACGGCGTCGAAGACAAA	TGACGGTCTCCACGGACAT
Pparγ	TCGCTGATGCACTGCCTATG	GAGAGGTCCACAGAGCTGATT
Ucp1	ACTGCCACACCTCCAGTCATT	CTTTGCCTCACTCAGGATTGG
Cidea	TGCTCTTCTGTATCGCCCAGT	GCCGTGTTAAGGAATCTGCTG
CD137	CGTGCAGAACTCCTGTGATAAC	GTCCACCTATGCTGGAGAAGG
Tmem26	ACCCTGTCATCCCACAGAG	TGTTTGGTGGAGTCCTAAGGTC
Tbx1	GGCAGGCAGACGAATGTTC	TTGTCATCTACGGGCACAAAG
Prdm16	CAGCACGGTGAAGCCATTC	GCGTGCATCCGCTTGTG
Occludin	ATGTCCGGCCGATGCTCTC	TTTGGCTGCTCTTGGGTCTGTAT
ZO-1	TTTTTGACAGGGGGAGTGG	TGCTGCAGAGGTCAAAGTTCAAG
Muc2	ACGTGTCATATTTGCACCTCT	TCAACATTGAGAGTGCCAACT
Reg3g	TTCCTGTCCTCCATGATCAAA	CATCCACCTCTGTTGGGTTC
Lyz1	GCCAAGGTCTACAATCGTTGTGAGTTG	CAGTCAGCCAGCTTGACACCACG
Pla2g2	AGGATTCCCCCAAGGATGCCAC	CAGCCGTTTCTGACAGGAGTTCTGG
Defa	GGTGATCATCAGACCCCAGCATCAGT	AAGAGACTAAAACTGAGGAGCAGC

Supplementary Figure S1:

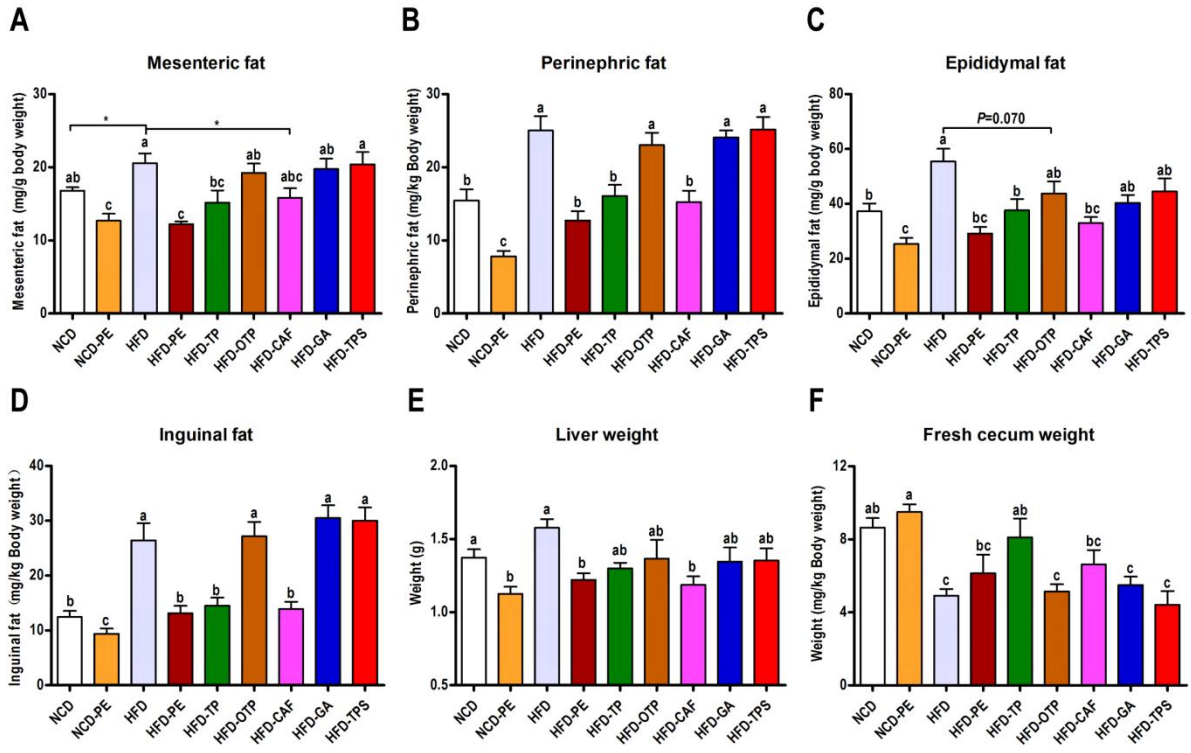

Figure S1 Effects of PEAC administration on the body composition of mice

Mice were fed either a NCD or a HFD for 12 weeks. Two groups of NCD-fed and HFD-fed mice were treated daily with oral doses of PE (750 mg/kg). The other five groups of HFD-fed mice were treated daily with oral doses of TP (250 mg/kg), OTP (250 mg/kg), TPS (250 mg/kg), CAF (50 mg/kg) and GA (50 mg/kg). The NCD- and HFD-fed control mice received a gavage with vehicle (water). Relative weight of mesenteric fat (A), perinephric fat (B), epididymal fat (C) and inguinal fat (D); (E) liver weight; (F) relative weight of fresh cecum. The data are expressed as the means ± SEM, n = 8-10. Data with different superscript letters are significantly different ($P<0.05$) according to the post hoc one-way ANOVA statistical analysis.

Supplementary Figure S2:

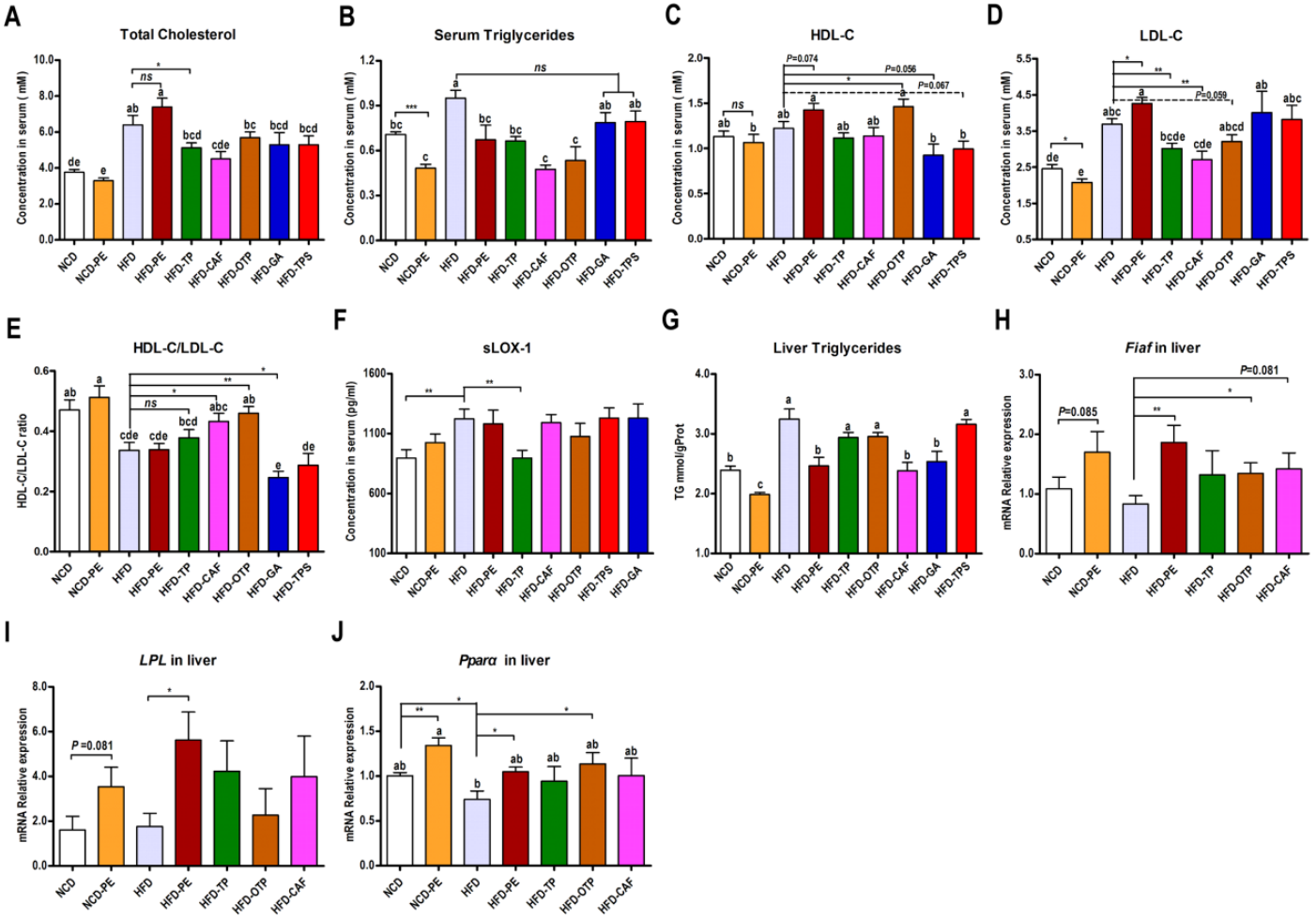

Figure S2 Effect of PEAC administration on lipid metabolism in the blood and liver

A-J: Effect of PEAC administration on lipid metabolism in the blood and liver. PE, TP, OTP and CAF improved the HFD-induced hyperlipidemia and liver adipose deposition to different extents. The blood lipid profile, including total cholesterol (A), TG (B), HDL-C (C), and LDL-C (D); (E) the ratio of HDL-C to LDL-C; (F) soluble (LOX-1) content, which is a receptor for ox-LDL; (G) TG content in the liver; mRNA expression of (H) *Fiaf*, (I) *LPL* and (J) *Pparα* in the liver.

The data are expressed as the means ± SEM, n = 8-10 (A-G); n = 6-8 (H-J). Data with different superscript letters are significantly different ($P<0.05$) according to the post hoc one-way ANOVA statistical analysis. * indicates a significant difference between two groups using the unpaired two-tailed Student t-test (* $P<0.05$, ** $P<0.01$).

Supplementary Figure S3:

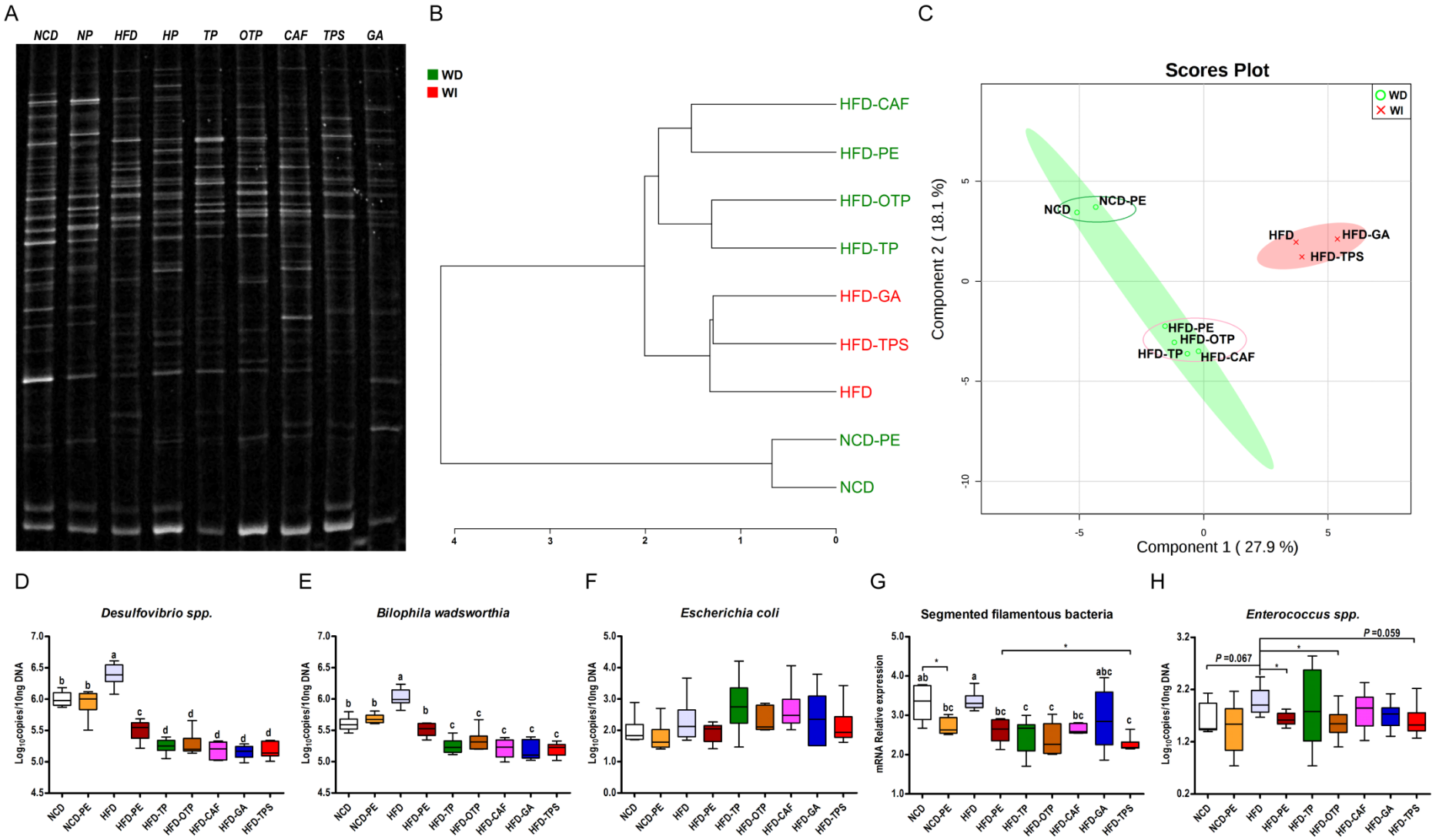

Figure S3 Effect of PEAC on the HFD-induced gut microbial community structure shift and selected specific gut bacteria

A-C: PEAC administration repaired the HFD-induced gut microbial community structural shift. Drastic changes in the microbial community structure in the cecal content of mice induced by PEAC administration and assessed by PCR-DGGE. (A) Microbial profiles of the cecal DNA content in different PEAC-treated mice with a denaturant gradient ranging from 42.5%-57.5%; (B) hierarchical clustering analysis (Pearson distance, Ward's clustering algorithm); (C) PLS-DA of different PEAC treatments; WI, weight increased groups compared to the HFD group; WD, weight decreased groups compared to the HFD group.

D-H: qPCR analyses of selected specific gut bacteria in mice cecal content. The data are expressed as Whisker plots from minimum to maximum, n = 6. Data with different superscript letters are significantly different ($P<0.05$) according to the post hoc one-way ANOVA statistical analysis. *indicates a significant difference between two groups using the unpaired two-tailed Student t-test (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

Supplementary Figure S4:

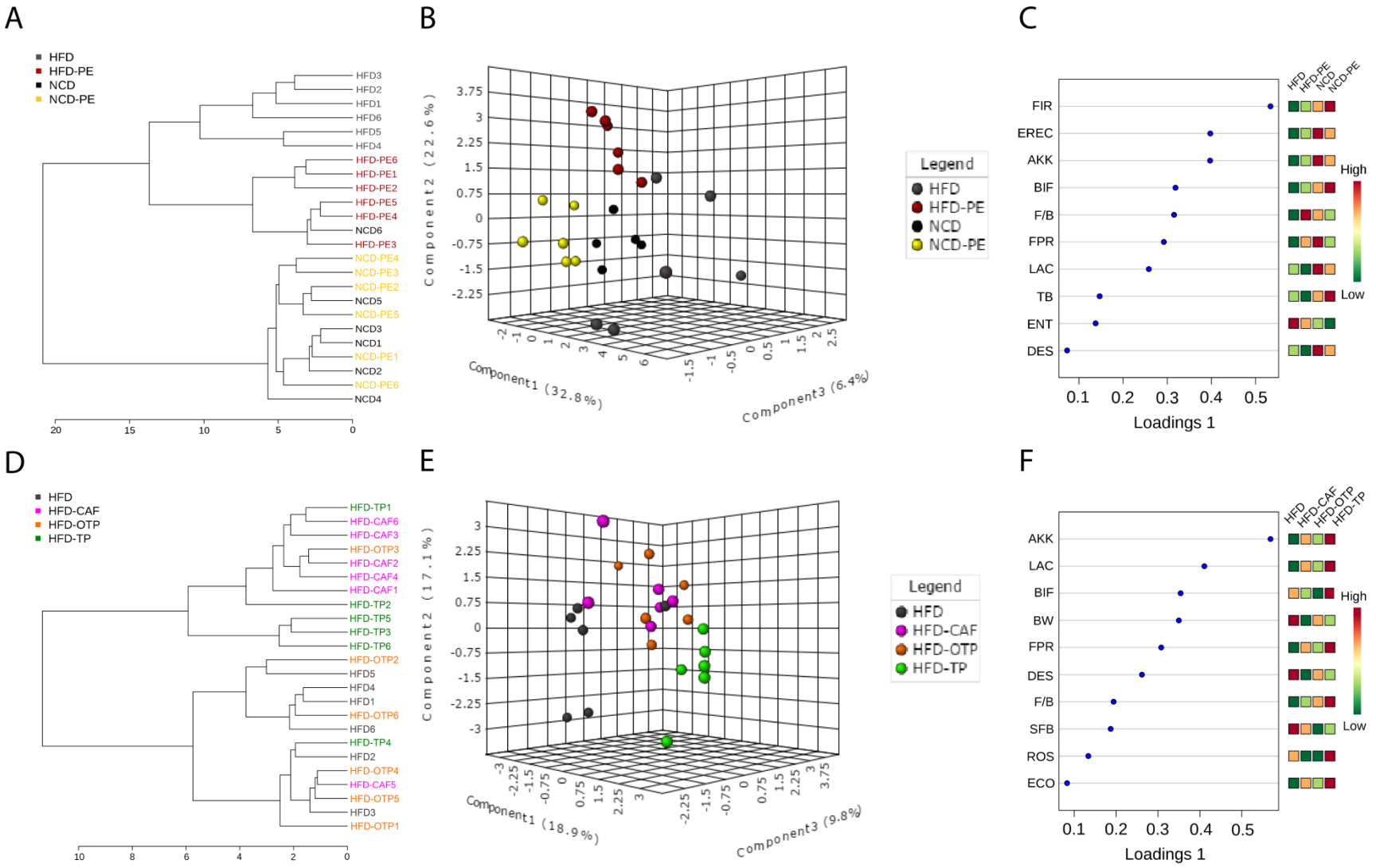

Figure S4 Multivariate statistical analysis using the qPCR results of selected gut bacteria in mouse ceca contents.

Hierarchical clustering analysis (Pearson distance, Ward's clustering algorithm) were performed with the results of NCD, NCD-PE, HFD and HFD-PE groups (A), or HFD, HFD-TP, HFD-OTP and HFD-CAF groups (D); sPLS-DA was performed to discriminate the key factors in the NCD, NCD-PE, HFD and HFD-PE group (B and C) or HFD, HFD-TP, HFD-OTP and HFD-CAF group (D and E).

Supplementary Figure S5:

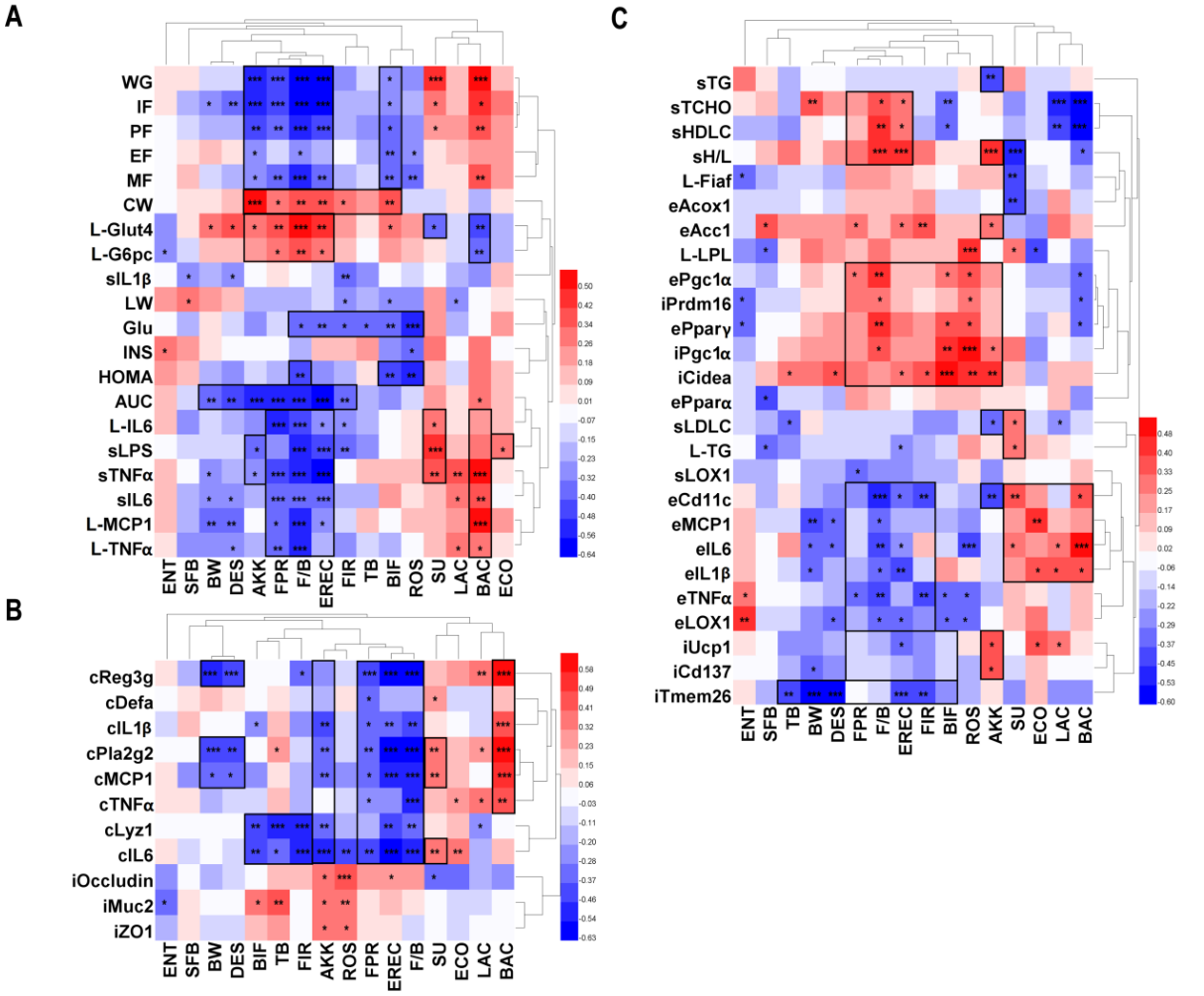

Figure S5 Biplot analysis and heat map correlation showing the associations among the bacterial taxa and host parameters.

Heat maps were generated to exhibit the Pearson correlations among all selected bacterial taxa in the qPCR analysis and all significantly affected host parameters by PEAC in the animal test. (A) Associations among bacterial taxa and host parameters, including body composition, metabolic endotoxemia, systemic and hepatic inflammation and glucose metabolism; (B) associations among bacterial taxa and intestinal homeostasis-related parameters; (C) associations among bacterial taxa and all lipid metabolism-related parameters and inflammatory factors in adipose tissue. *P*-values were adjusted for multiple testing

according to the Bonferroni and Hocheberg procedures. The color at each intersection indicates the value of the *r* coefficient; * indicates a significance correlation between these two parameters (*P<0.05, **P<0.01, ***P<0.001).

TB, total bacteria; FIR, *Firmicutes*; BAC, *Bacteroidetes*; F/B, *Firmicutes*/*Bacteroidetes*; AKK, *Akkermansia muciniphila*; EREC, *Eubacterium rectale*/*Clostridium coccoides* group; FPR, *Faecalibacterium prausnitzii*; ROS, *Roseburia* spp.; BIF, *Bifidobacterium* spp.; *Lactobacillus* spp. (LAC); SFB, Segmented filamentous bacteria; SU, *Sutterella* spp.; BW, *Bilophia wadsworthia*; DES, *Desulfovibrio* spp.; ENT, *Enterococcus* spp.; ECO, *Escherichia coli.*

On the left side of Figure 7A: WG, body weight gain; IF, weight of inguinal fat; PF, weight of perinephric fat; EF, weight of epididymal fat; MF, weight of mesenteric fat; CW, cecum weight; LW, liver weight. L-TNFα indicates *TNFα* expressed in the liver; sLPS indicates LPS expressed in the serum and other indexes.

On the left side of Figure 7B: cTNFα indicates *TNF-α* expressed in the colon; iZO1 indicates *ZO-1* expressed in the ileum and other indexes.

On the left side of Figure 7C: sH/L indicates the HDL-C/LDL-C value in the serum; sTG indicates the TG content in the serum; L-TG indicates the TG content in the liver; eTNFα indicates *TNF-α* expressed in the epididymal fat pad; iUcp1 indicates *Ucp1* expressed in the inguinal fat pad and other indexes.

Supplementary Figure S6:

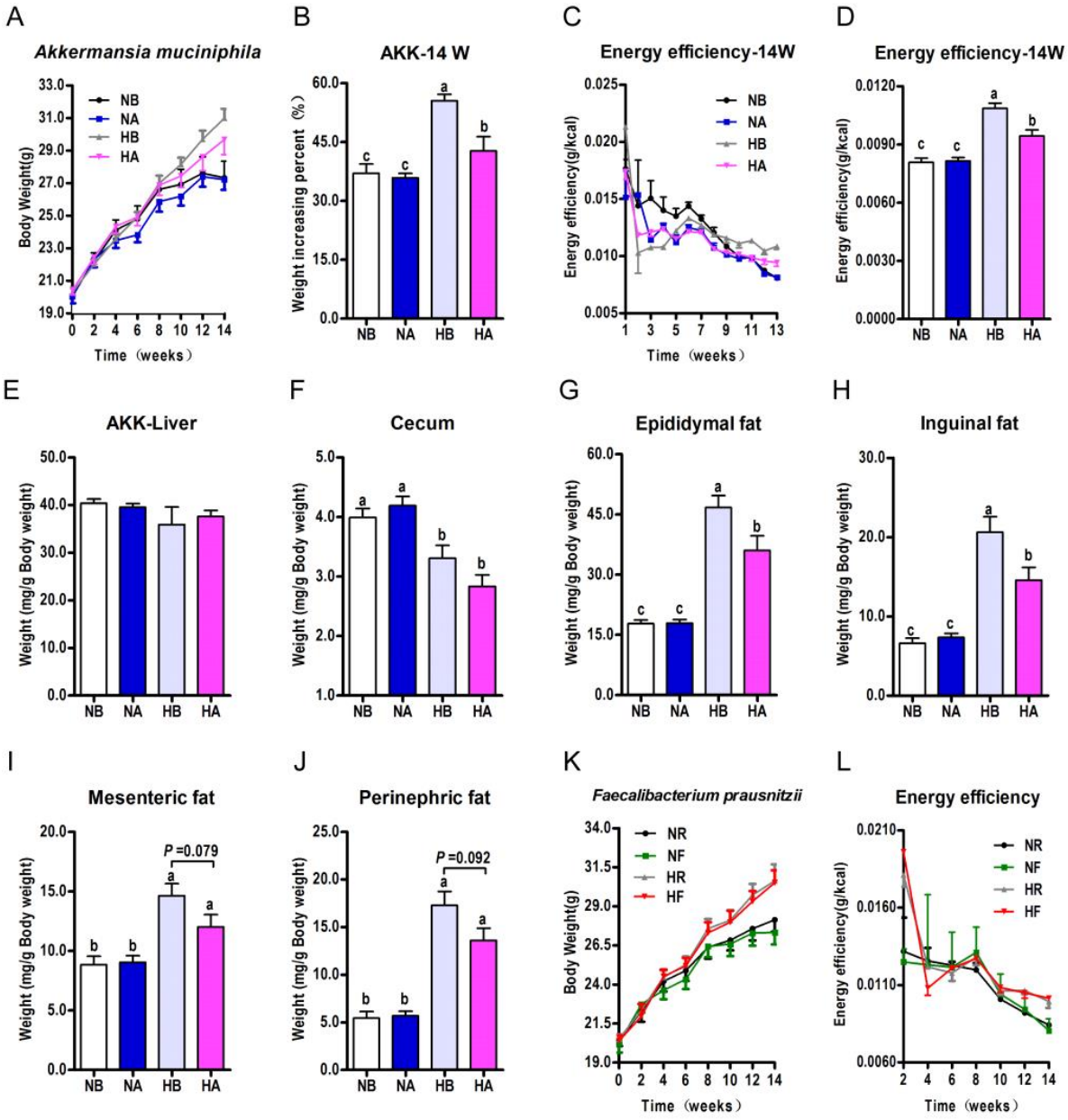

Figure S6 Effects of AKK and FPR administrations on body composition and energy efficiency of mice.

Six-week-old mice were fed either a NCD or a HFD for 14 weeks. Two groups of NCD-fed and HFD-fed mice were treated daily with oral doses of 300 μl AKK culture medium (1×10^9 cfu/ml liquid; NA, HA) or 300 μl FPR culture medium (2×10^9 cfu/ml liquid; NF, HF). NCD- and HFD-fed control mice received a gavage with the corresponding sterile culture medium (AKK: NB, HB; FPR: NR, HR).

(A, K) Weight gain curves; (B) total weight gain; (C, D, L) energy efficiency (ie, ratio between body weight gain and energy intake); Relative weight of liver (E), cecum (F), epididymal fat (G), and inguinal fat (H); mesenteric fat (I), perinephric fat (J). The data are expressed as the means ± SEM, n = 8-10. Data with different superscript letters are significantly different ($P<0.05$) according to post hoc ANOVA one way statistical analysis. *P* values in figures indicate the difference between two groups using the unpaired two-tailed Student t-test.

Supplementary Figure S7:

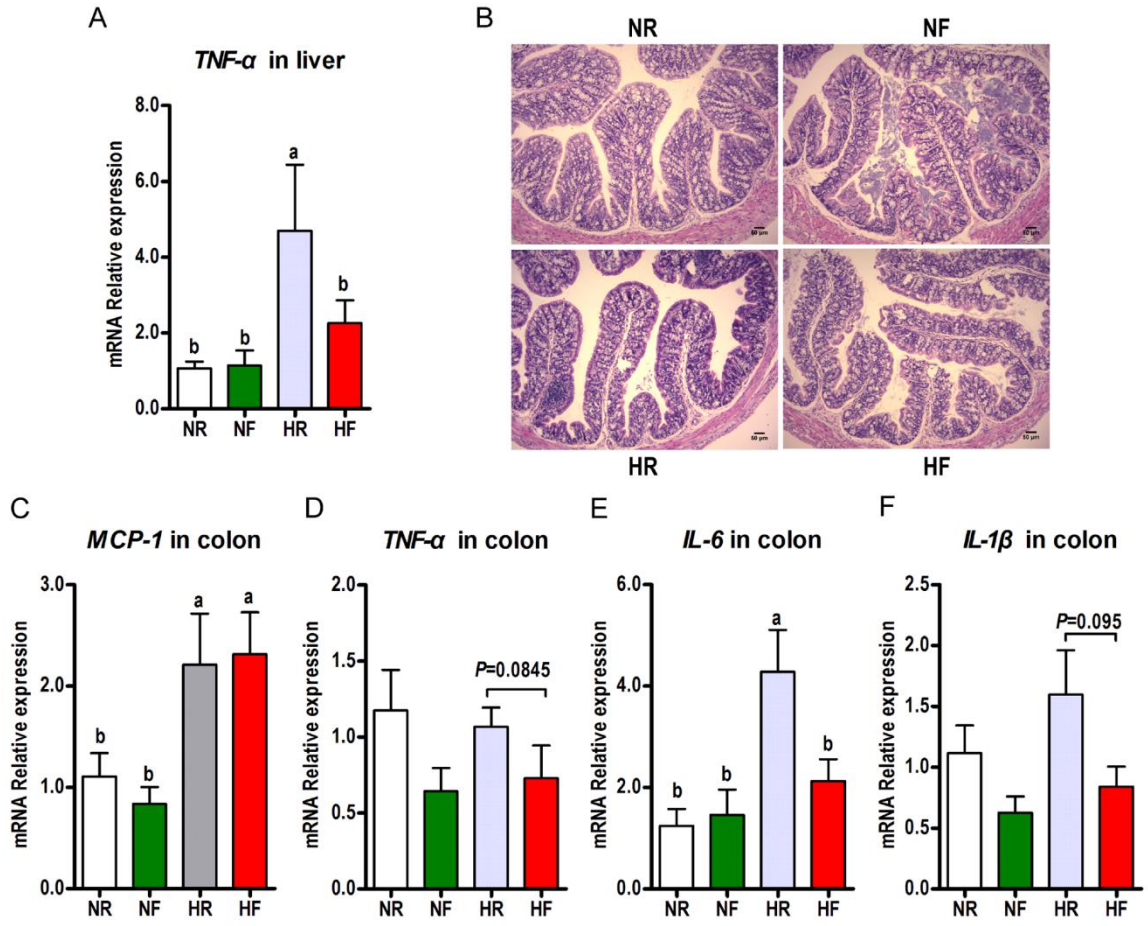

Figure S7 FPR administrations reduced the mRNA expression of makers of inflammation in liver and colon.

Six-week-old mice were fed either a NCD or a HFD for 14 weeks. Two groups of NCD-fed and HFD-fed mice were treated daily with oral doses of 300 μl FPR culture medium (2×10^9 cfu/ml liquid; NF, HF). NCD- and HFD-fed control mice received a gavage with the corresponding sterile culture medium (NR, HR).

(A) mRNA expression of *TNF-α* in liver; (B) Photomicrographs of H&E-stained proximal colon sections. Infiltration of inflammatory cells into tissue gives bluer photomicrographs in the HR group; Expression of inflammation gene in proximal colon, including (C) *MCP-1*, (D) *TNF-α*, (E) *IL-6* and (F) *IL-1β*. The data are expressed as the means ± SEM $n = 6$. Data with different superscript letters are significantly different ($P<0.05$) according to post hoc ANOVA one way statistical analysis. *P* values in figures indicate the difference between two groups using the unpaired two-tailed Student t-test.

Supplementary Figure S8:

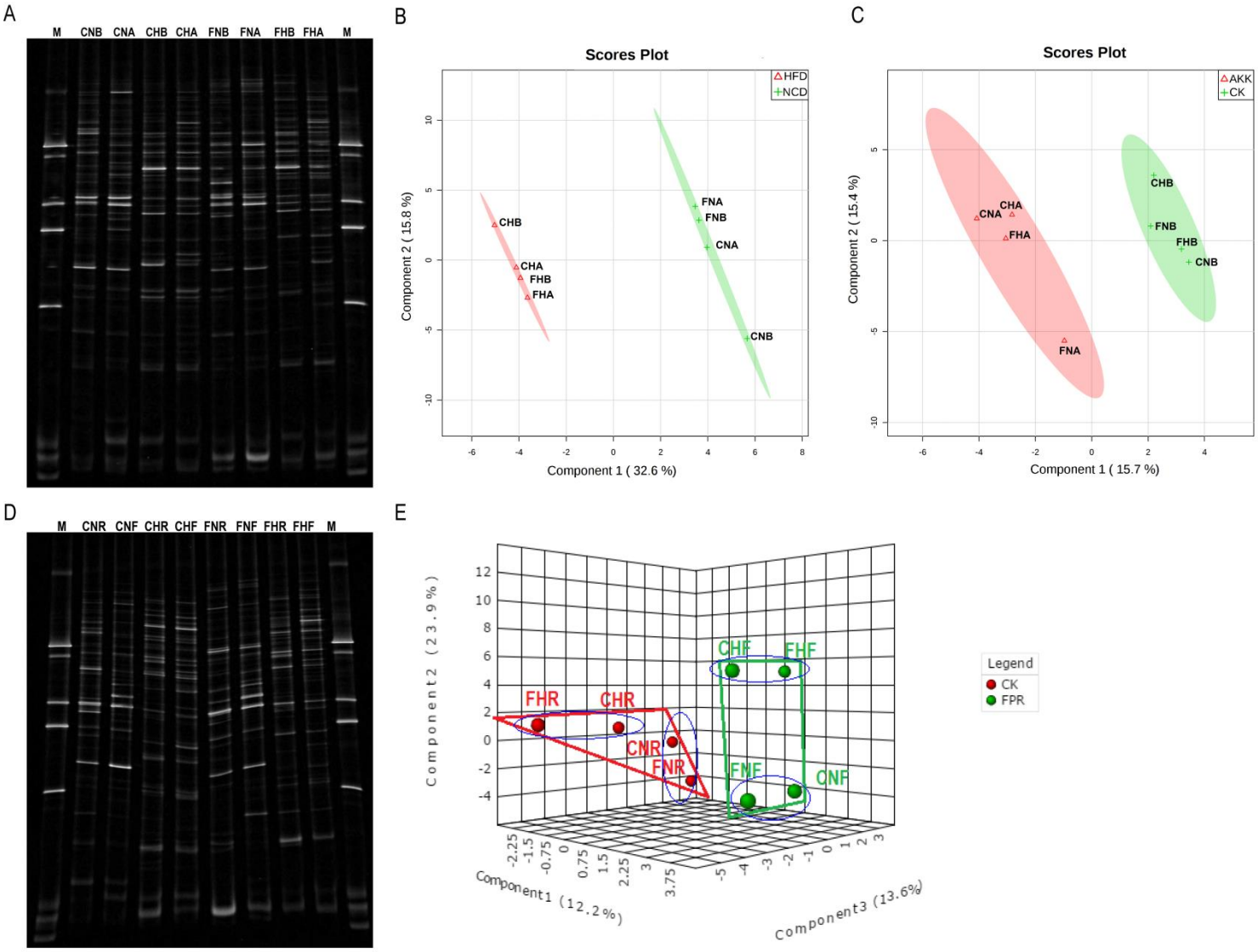

Figure S8 AKK and FPR administrations altered the gut microbial community structure

Six-week-old mice were fed either a NCD or a HFD for 14 weeks. Two groups of NCD-fed and HFD-fed mice were treated daily with oral doses of 300 μl AKK culture medium (1×10^9 cfu/ml liquid; NA, HA) or 300 μl FPR culture medium (2×10^9 cfu/ml liquid; NF, HF). NCD- and HFD-fed control mice received a gavage with the corresponding sterile culture medium (AKK: NB, HB; FPR: NR, HR).

Changes in microbial community structure in cecal content and feces of mice induced by AKK and FPR administration and assessed by PCR-DGGE. Microbial profiles of cecal content and feces DNA of AKK (A) and FPR (D) treated mice with a denaturant gradient ranging from 42.5%-57.5%; CNB, CNA, CHB and CHA in figure A indicate cecal content of NB, NA, HB and HA group; CNR, CNF, CHR and CHF in figure D indicate cecal content of NR, NF, HR and HF group. FNB, FNA, FHB and FHA in figure A indicate feces of NB, NA, HB and HA group; FNR, FNF, FHR and FHF in figure D indicate feces content of NR, NF, HR and HF group. (B) Partial least squares-discriminant analysis (PLS-DA) from the aspect of diet types (NCD vs HFD); (C) PLS-DA from the aspect of AKK administration or not (CK vs AKK); (E) PLS-DA from the aspect of FPR administration or not (CK vs FPR).

Supplementary Figure S9:

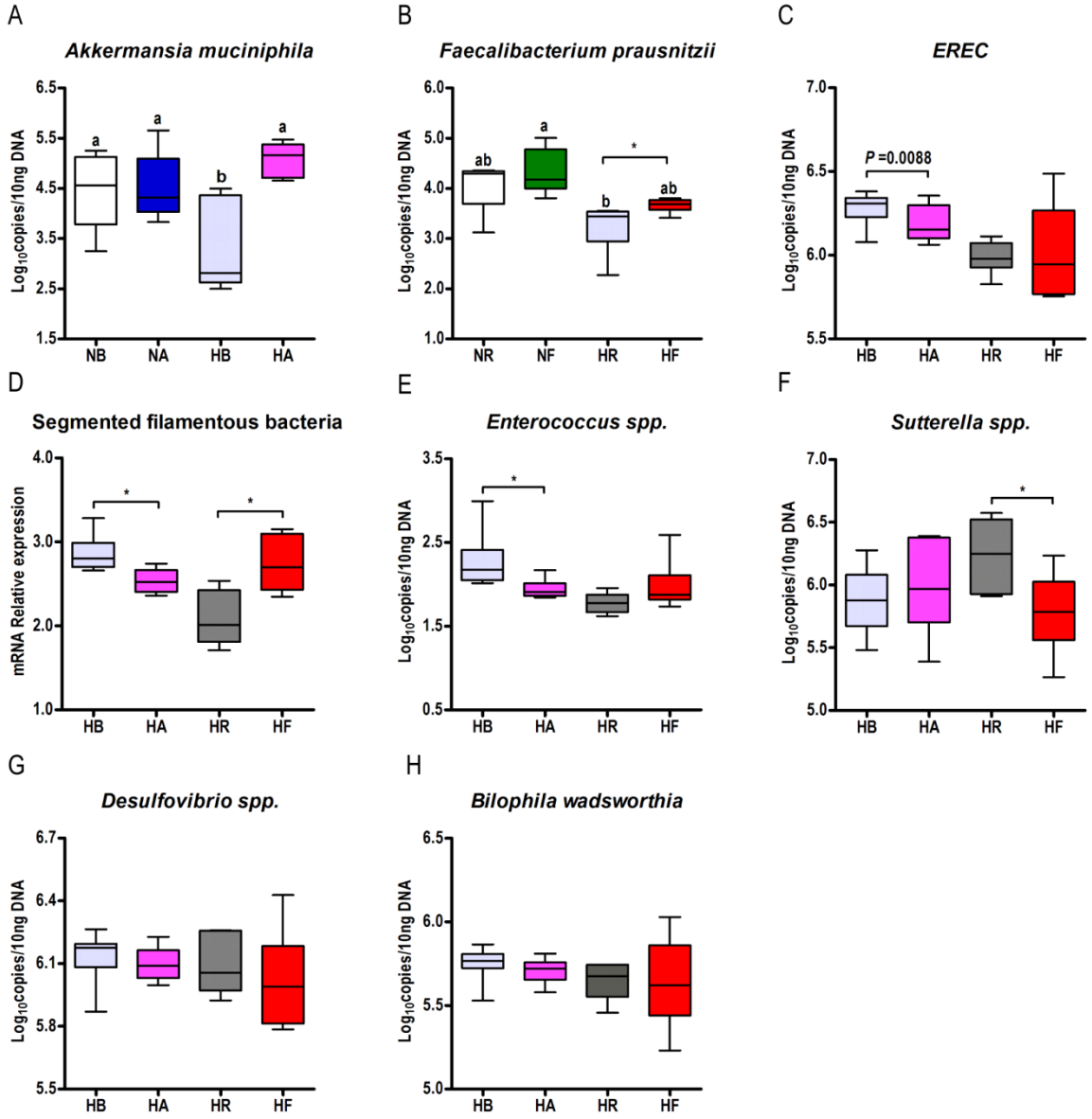

Figure S9 Effects of AKK and FPR administrations on selected specific gut bacteria in mouse cecal content

Six-week-old mice were fed either a NCD or a HFD for 14 weeks. Two groups of NCD-fed and HFD-fed mice were treated daily with oral doses of 300 µl AKK culture medium (1×109 cfu/ml liquid; NA, HA) or 300 µl FPR culture medium (2×109 cfu/ml liquid; NF, HF). NCD- and HFD-fed control mice received a gavage with the corresponding sterile culture medium (AKK: NB, HB; FPR: NR, HR).

(C) Eubacterium rectale/Clostridium coccoides group (EREC). The data are expressed as Whiskers plots with minimum to maximum, n = 6-8. Data with different superscript letters are significantly different ($P<0.05$) according to post hoc ANOVA one way statistical analysis. *indicates a significant difference between two groups using the unpaired two-tailed Student t-test ($P<0.05$).