Modulation of Gut Microbiota during Probiotics-Mediated Attenuation of Metabolic Syndrome in High Fat Diet-Fed Mice

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Supplementary Information

Summary

The supplementary information includes supplementary materials and methods, eleven supplementary figures and four supplementary tables.

Supplementary Materials and Methods

Animal trial

A. Mice

Forty male SPF grade C57BL/6J mice at 10 weeks of age, with a weight between 22.5 to 26.5g, were purchased from National Rodent Laboratory Animal Resources. Mice were kept under stable conditions with a 12-hour daylight cycle, a temperature of 22 ± 3 ℃, and free access to water and food in accredited animal facilities of Shanghai Laboratory Animal Center (SLAC).

All animal procedures and protocols were performed in accordance with institutional guidelines of SLAC and with approval from the institutional animal care and use committee of SLAC.

B. Bacteria suspension preparation

The three strains were cultured with MRS broth (OXOID, Basingstoke, UK) at 37 ℃ to reach early stationary phase. The cultures were centrifuged, and bacteria cells were resuspended with fresh MRS broth to 10^8 colony-forming units (CFUs) / 200μl, stored at -80 ℃ and each aliquot was thawed 1 hour before it was administered to each mouse by gavage. The number of the bacterial cells was determined by plating the serial dilutions of the inoculum suspensions on MRS agar plates.

C. Sample collection

Animal treatments lasted for 12 weeks, during which the body weight of each mouse and food intake of every cage of mice (four mice per cage) were measured once a week. Stool samples were collected from each of the 40 mice (8 mice per group) at baseline and 12th week by keeping individual animals in a metabolic cage for 8 hours, and immediately stored at -80 ℃ for subsequent microbiota analysis. The feces at each time points was collected on two adjacent days, and stools of four mice from each group were collected on each day.

To monitor the fecal recovery of the strains, three mice were randomly selected in each group at 2nd, 6th and 11th week after the start of the probiotic administration, and fecal samples from these mice were collected, and stored at -80 ℃ for RNA extraction and subsequent quantification of the strain.

At the end of the 12th week, after 5 h of food deprivation, all blood was collected from the orbital plexus, and serum was isolated by centrifugation at 3000 rpm at 4 ℃ for 15 min and stored at -80 ℃ for subsequent biochemical testing. All animals were sacrificed by cervical dislocation. eAT, liver and jejunum were excised. For liver, the free end of the largest lobe was sampled in RNALater (Ambion, Austin, TX, USA), and the fixed end in paraformaldehyde. The left depot of epididymal adipose tissue was longitudinally cut into two halves, the middle part of the right half was sampled in RNALater, and the left half in paraformaldehyde. For jejunum, 2 cm of intestine tissue 1.5 cm away from the stomach pylori was excised and kept in RNALater. These samples were collected from each mouse. Cecal content was collected and snap frozen in liquid nitrogen, and then stored at -80 ℃ until analysis.

D. **Quantification of the probiotic strains in feces**

Total RNA was extracted from 20 mg fresh fecal sample by a modified acid guanidinium thiocyanate-phenol-chloroform extraction method as described previously (Matsuda *et al.*, 2007) and submitted to reverse transcription (RT)-quantitative (q) PCR.

RT-qPCR was performed using an OneStep RT-PCR kit (QIAGEN, Hilden, Germany) according to manufacturer’s instructions on a DNA Engine OPTICON2 continuous Fluorescence Detector (MJ research, Waltham, MA, USA). Primers are listed in Supplementary Table S2. Data were collected and analyzed using MJ Opticon Monitor Analysis Software accompanying the PCR machine.

E. Oral glucose tolerance test (OGTT)

After 5 h of food deprivation, glucose was administered orally to the mice at a dose of 2.0 g/kg body weight. Blood samples were taken from the tail before and 15, 30, 60, and 120 min after glucose administration, and blood glucose levels were measured with a blood glucose meter (Accu-Check; Roche Diagnostics, Mannheim, Germany). The blood glucose level before glucose administration represented fasting glucose level.

Histomorphology and immunohistochemistry

Digital images of hematoxylin and eosin-stained sections were acquired with an Olympus DX51 light microscope. Adipocyte size (cross-sectional area) was obtained from perimeter tracings using Image J software (Sun Microsystems, Mountain View, CA, USA). For each mouse, cell areas were determined in at least two histologic sections cut 50 μm apart (>500 total adipocytes). Immunohistochemistry was performed using VectaStain kits (Vector Labs, Burlingame, CA, USA). Primary antibodies were rat anti-mouse Mac-2 (cat. # CL8942AP, Cedarlane Labs, Ontario, Canada) and goat anti-mouse MMP-12 (Santa Cruz Labs, Santa Cruz, CA, USA). Isotype-matched nonimmune IgG or peptide-neutralized primary antibody served as negative controls. All morphometric and immunohistochemistry studies were performed by individuals who were 'blinded' to the sample treatments.

Quantification of host gene expression

Total RNA was extracted from about 100 mg eAT, 30 mg liver and 40 mg jejunum using RNeasy® lipid tissue mini kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. RNA concentrations were measured using the NanoVue spectrophotometer (GE Healthcare, Waukesha, WI, USA) and the integrity was checked by denaturing agarose gel electrophoresis.

Contaminating DNA was removed using the DNase I (Invitrogen Life Technologies, Carlsbad, CA, USA) digestion according to the manufacturer' s instructions, and DNA contamination was tested by PCR with primer targeting housekeeping gene GAPDH. RNA concentrations were measured again. Complementary DNA (cDNA) was generated from 500 ng of high-quality total RNA with SuperScript™ III First-Strand synthesis system (Invitrogen Life Technologies, Carlsbad, CA, USA).

Real-time quantitative PCR was performed with the iQ™ SYBR® Green Surpermix (BIO-RAD, Hercules, CA, USA) on a DNA Engine OPTICON2 continuous Fluorescence Detector (MJ research, Waltham, MA, USA). Data were collected and analyzed using MJ Opticon Monitor Analysis Software accompanying the PCR machine. All mRNA quantification data were normalized to GAPDH. Gene expression levels were expressed as values relative to the mouse group fed on normal chow.

Bioinformatics and statistical analysis of 454 pyrosequencing data

High-quality reads for bioinformatics analysis were selected by processing the raw data as following: 1) search the primers by using blast-based matching (Word size=4, E-value=0.1), the primer at least at the sequencing end should exist; 2) locate the barcodes according to the position of the primers; reads should have at least one complete barcode; reads without entire barcodes at both ends, or with complete but poor-matched barcode pairs (more than one insertion / deletion / mismatch) were discarded; 3) according to the complete barcode (if the barcodes were complete at both ends and they were mismatched, take the barcode at the sequencing end), assign the read to the corresponding sample; 4) after trimming the primer and barcode bases, those sequences with variable region more than 100 nt and less than 300 nt in length and no more than two undetermined bases were preserved.

All high-quality sequences were aligned by Nearest Alignment Space Termination (NAST) multi-aligner with template length $\geq$ 90 bases and percent identity $\geq$ 75% in Greengenes database, and then clustered using CD-HIT with 100% similarity. The most abundant sequence of each cluster was selected as the representative of unique sequence, and then searched against the RDP database (RDP Classifier) at 50% confidence level to determine the phylogeny. The representative sequences were also imported into the ARB to construct a neighbor-joining tree. OTUs were classified with DOTUR at 98% similarity level. Rarefaction analysis and Shannon diversity index were calculated using QIIME (Caporaso *et al.*, 2010). Principal component analysis (PCA) was performed on relative abundances (normalized for each sample) of OTUs, and weighted Fast UniFrac principal coordinate analysis (PCoA) was done with the phylogenetic tree constructed by inserting the representative of each OTU into pre-established phylogenetic trees of full-length 16S rRNA gene sequences in ARB. The statistical significance of the separation among animal groups in PCoA scores plots was assessed by multivariate analysis of variance (MANOVA) test with MATLAB R2010a (The MathWorks, Inc., Natick, MA, USA). The relative abundance of each OTU was log-transformed, and used to construct RDA models to find the OTUs that were different between two animal groups with Canoco for Windows 4.5 (Microcomputer Power, Ithaca, NY, USA) according to the manufacturer's instructions. Statistical significance was assessed by Monte Carlo Permutation Procedure (MCPP) with 499 random permutations under the full model.

Cecal fermentation end products measurement

Two milliliters supernatant was prepared by reconstituting all cecal content of each animal in 0.01M phosphate buffer solution (PBS) followed by centrifugation at 9000g for 5 min at 4 ℃. The supernatant was acidified with a 1/10 volume of 50% H_2SO_4 and extracted with ethyl ether. The concentrations of SCFAs and BCFAs were determined in the organic phase using an Agilent 6890N gas chromatograph (Agilent Technologies, Wilmington, DE, USA) equipped with a polar HP-FFAP capillary column (0.25 mm × 0.25 mm × 30 m) and flame ionization detector (Agilent Technologies, Wilmington, DE, USA). Helium was used as the carrier gas. The initial oven temperature was 120 ℃, which was maintained for 16 min and then raised to 122 ℃ at 5 ℃ / min, increased to 250 ℃ at 30 ℃ / min, and held at this temperature for 3 min. The detector temperature was 270 ℃, and the injector temperature was 260 ℃. Data handling was performed with an Agilent ChemStation (version G2070AA, Agilent Technologies, Wilmington, DE, USA).

Supplementary Tables

Table S1 Compositions of experimental diets

Ingredients	Normal chow diet (Research Diets D12450B)	High fat diet (Research Diets D12492)
	g / 100 g diet	
Casein, 80 Mesh	18.96	25.85
L-Cystine	0.28	0.39
Corn Starch	29.86	0.00
Maltodextrin 10	3.32	16.15
Sucrose	33.18	8.89
Cellulose, BW200	4.74	6.46
Soybean Oil	2.37	3.23
Lard	1.90	31.66
Mineral Mix, S10026	0.95	1.29
DiCalcium Phosphate	1.23	1.68
Calcium Carbonate	0.52	0.71
Potassium Citrate,1 H_2O	1.56	2.13
Vitamin Mix, V10001	0.95	1.29
Choline Bitartrate	0.19	0.26

Table S2 List of primers used in this study

Target	Sequences (5’-3’)*	Product size (bp)	Annealing temperature (℃)	References
Bacterial 16S rRNA gene				
V3 region	F: NNNNNNNNCCTACGGGAGGCAGCAG R: NNNNNNNNATTACCGCGGCTGCT	About 200	65-55 (touch down)	(Zhang *et al.*, 2010)
Lactobacillus paracasei (LC) / *Lactobacillus rhamnosus* (LR)	F : ACCGCATGGTTCTTGGC R: CCGACAACAGTTACTCTGCC	296	60	(Matsuda *et al.*, 2009)
Bifidobacterium animalis subp. *lactis* (BA)	F: CCCTTTCCACGGGTCCC R: AAGGGAAACCGTGTCTCCAC	194	65	(Matsumoto *et al.*, 2009)
Mouse genes				
GAPDH	F: GTGTTCCTACCCCCAATGTGT R: ATTGTCATACCAGGAAATGAGCTT	248	55	(Masui *et al.*, 2007)
CD11c	F: CTGGATAGCCTTTCTTCTGCTG R: GCACACTGTGTCCGAACTC	113	55	(Lumeng *et al.*, 2007)
TNFα	F : ACGGCATGGATCTCAAAGAC R: AGATAGCAAATCGGCTGACG	138	55	(Chiang *et al.*, 2009)
MCP-1	F: TTAAAAACCTGGATCGGAACCAA R: GCATTAGCTTCAGATTTACGGGT	121	55	(Chiang *et al.*, 2009)
adiponectin	F: AGGTTGGATGGCAGGC R: GTCTCACCCTTAGGACCAAGAA	129	55	(Shibata *et al.*, 2007)
leptin	F: CCTGTGGCTTTGGTCCTATCTG R: AGGCAAGCTGGTGAGGATCTG	244	55	(Klaus *et al.*, 2005)

*The NNNNNNNN was the unique 8-base barcode which was used to sort PCR products into different samples.

Table S3 The number of sequences lost during the bioinformatics analysis of the 454 pyrosequencing run containing the 80 fecal samples of the present study[a]

Sequencing defects	Number of sequences lost (percentage accounting to the total sequences of the run)
Sequences with unqualified primers [b]	3606 (0.57%)
Sequences with unqualified barcode [c]	127154 (20.26%)
Sequences with too short variable region (<90bp)	526 (0.08%)
Sequences with undetermined bases in variable region (>2 bases)	4 (0.0006%)
Total	131290 (20.93%)

[a], Totally 148 samples were sequenced in this 454 run, and 627349 reads were obtained.

[b], The primer for the 16S rRNA gene V3 region at the sequencing end could not be found in the sequences.

[c], In these sequences, complete barcode could not be found at neither ends, or barcodes at both ends was poor-matched with more than one insertion / deletion / mismatch, or barcodes that did not exist in the barcode table were found.

Table S4 The phylogeny and relative abundance of the 83 OTUs altered by probiotics, and the p value of Mann-Whitney test evaluating the abundance difference of these OTUs between each probiotic group and HFD group. In grey are underlined the significant p values (P < 0.05)

OTU ID	Taxonomical assignments (RDP Classifier)			Relative abundance (%) Median (minimum, maximum)					P values calculated by Mann-Whitney test			
	Phylum	Family	Genus	HFD+LC	HFD+LR	HFD+BA	HFD	NC	HFD+LC vs. HFD	HFD+LR vs. HFD	HFD+BA vs. HFD	NC vs. HFD
OTU0379	Actinobacteria	*Bifidobacteriaceae*	*Bifidobacterium*	0.00 (0.00, 0.11)	0.00 (0.00, 0.00)	0.01 (0.00, 0.23)	0.00 (0.00, 0.00)	0.47 (0.00, 3.21)	1.0000	1.0000	0.0769	0.0014
OTU0173	Actinobacteria	*Coriobacteriaceae*	*Olsenella*	0.01 (0.00, 0.51)	0.60 (0.41, 1.58)	0.26 (0.00, 0.50)	0.01 (0.00, 0.22)	0.47 (0.03, 1.86)	0.8629	0.0003	0.0258	0.0042
OTU0109	Bacteroidetes	*Porphyromonadaceae*	*Barnesiella*	0.74 (0.00, 1.33)	0.21 (0.03, 1.80)	0.41 (0.00, 1.75)	0.00 (0.00, 0.00)	1.04 (0.10, 2.78)	0.0014	0.0003	0.0014	0.0002
OTU0002	Bacteroidetes	*Porphyromonadaceae*	*Barnesiella*	0.05 (0.00, 0.18)	0.03 (0.00, 0.08)	0.01 (0.00, 0.11)	0.00 (0.00, 0.07)	0.11 (0.00, 0.93)	0.0373	0.4615	0.5301	0.0068
OTU0054	Firmicutes	*Erysipelotrichaceae*	*Allobaculum*	1.23 (0.00, 3.76)	2.41 (1.36, 4.95)	1.30 (0.05, 2.47)	0.58 (0.00, 2.25)	6.96 (3.34, 19.13)	0.3807	0.0022	0.1304	0.0002
OTU0073	Firmicutes	*Erysipelotrichaceae*	*Allobaculum*	0.19 (0.00, 0.45)	3.42 (1.00, 8.64)	0.71 (0.00, 4.01)	0.00 (0.00, 0.61)	2.72 (0.17, 6.78)	0.2668	0.0003	0.0200	0.0003
OTU0059	Firmicutes	*Erysipelotrichaceae*	*Allobaculum*	0.00 (0.00, 0.37)	0.00 (0.00, 0.16)	0.00 (0.00, 0.05)	0.00 (0.00, 0.00)	1.43 (0.18, 4.89)	0.4667	0.1538	1.0000	0.0002
OTU0455	Firmicutes	*Ruminococcaceae*		0.00 (0.00, 0.09)	0.00 (0.00, 0.15)	0.06 (0.00, 0.20)	0.00 (0.00, 0.09)	0.06 (0.00, 0.13)	0.8564	0.4615	0.0623	0.0145
OTU0043	Firmicutes	*Lachnospiraceae*		0.35 (0.00, 0.58)	0.00 (0.00, 0.00)	0.00 (0.00, 0.10)	0.00 (0.00, 0.00)	0.05 (0.00, 0.37)	0.0014	1.0000	1.0000	0.0070
OTU0306	Firmicutes	*Lachnospiraceae*		0.17 (0.00, 0.72)	0.21 (0.00, 0.84)	0.51 (0.00, 0.71)	0.00 (0.00, 0.03)	0.55 (0.05, 1.83)	0.0126	0.0028	0.0256	0.0002
OTU0025	Bacteroidetes	*Porphyromonadaceae*	*Barnesiella*	2.63 (1.96, 3.70)	3.65 (1.01, 6.11)	1.71 (0.61, 3.20)	2.03 (0.96, 3.03)	0.63 (0.03, 1.17)	0.0499	0.0205	1.0000	0.0070
OTU0190	Bacteroidetes	*Porphyromonadaceae*	*Barnesiella*	0.02 (0.00, 0.14)	0.21 (0.03, 0.57)	0.04 (0.00, 0.27)	0.01 (0.00, 0.29)	0.00 (0.00, 0.00)	1.0000	0.0482	0.6291	0.0769
OTU0132	Bacteroidetes	*Porphyromonadaceae*	*Barnesiella*	0.29 (0.14, 1.79)	0.66 (0.11, 2.19)	0.63 (0.07, 1.35)	0.23 (0.11, 0.43)	0.00 (0.00, 0.02)	0.2345	0.0401	0.0281	0.0002
OTU0536	Firmicutes	*Erysipelotrichaceae*	*Erysipelotrichaceae_incertae_sedis*	0.32 (0.03, 0.61)	0.00 (0.00, 0.18)	0.11 (0.00, 0.45)	0.09 (0.00, 0.20)	0.00 (0.00, 0.03)	0.0274	0.4289	0.6402	0.0126
OTU0011	TM7	*TM7_genera_incertae_sedis*	*TM7_genera_incertae_sedis*	0.36 (0.28, 0.88)	0.13 (0.00, 0.76)	0.27 (0.00, 0.78)	0.21 (0.07, 0.52)	0.15 (0.05, 0.62)	0.1304	0.8665	0.8785	0.5737
OTU0605	Actinobacteria	*Coriobacteriaceae*	*Enterorhabdus*	0.00 (0.00, 0.06)	0.03 (0.00, 0.08)	0.00 (0.00, 0.03)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	0.4667	0.0513	0.4667	1.0000
OTU0055	Bacteroidetes	*Bacteroidaceae*	*Bacteroides*	2.73 (0.68, 4.54)	1.03 (0.44, 1.46)	1.61 (0.68, 5.98)	1.03 (0.25, 2.29)	1.72 (0.35, 3.06)	0.0650	0.7789	0.1949	0.2345
OTU0556	Bacteroidetes	*Prevotellaceae*	*Paraprevotella*	0.15 (0.05, 0.23)	0.06 (0.03, 0.28)	0.05 (0.02, 0.22)	0.04 (0.00, 0.49)	0.00 (0.00, 0.39)	0.0145	0.2222	0.2670	0.3049
OTU0399	Bacteroidetes	*Prevotellaceae*	*Paraprevotella*	0.06 (0.05, 0.15)	0.00 (0.00, 0.10)	0.02 (0.00, 0.16)	0.04 (0.00, 0.16)	0.00 (0.00, 0.12)	0.1588	0.2723	0.8757	0.1002
OTU1290	Bacteroidetes	*Porphyromonadaceae*	*Parabacteroides*	0.01 (0.00, 0.29)	0.00 (0.00, 0.00)	0.00 (0.00, 1.00)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	0.0769	1.0000	0.2000	1.0000
OTU0007	Bacteroidetes	*Porphyromonadaceae*	*Barnesiella*	0.85 (0.23, 2.45)	0.26 (0.00, 1.80)	0.11 (0.00, 1.47)	0.00 (0.00, 0.49)	0.03 (0.00, 0.73)	0.0011	0.0528	0.2668	0.5301
OTU0037	Bacteroidetes	*Porphyromonadaceae*	*Barnesiella*	0.00 (0.00, 0.03)	0.03 (0.03, 0.15)	0.00 (0.00, 0.11)	0.00 (0.00, 0.07)	0.00 (0.00, 0.07)	0.7333	0.0099	0.8564	1.0000

OTU1132	Bacteroidetes	*Porphyromonadaceae*	*Barnesiella*	0.00 (0.00, 0.00)	0.00 (0.00, 0.13)	0.00 (0.00, 0.02)	0.00 (0.00, 0.00)	0.00 (0.00, 0.03)	1.0000	0.1538	1.0000	1.0000
OTU0071	Bacteroidetes	*Porphyromonadaceae*	*Barnesiella*	0.00 (0.00, 0.13)	0.09 (0.00, 0.32)	0.00 (0.00, 0.08)	0.00 (0.00, 0.13)	0.00 (0.00, 0.06)	1.0000	0.0155	0.7333	0.7333
OTU1472	Bacteroidetes	*Porphyromonadaceae*	*Barnesiella*	0.00 (0.00, 0.00)	0.00 (0.00, 1.79)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	1.0000	0.1538	1.0000	1.0000
OTU2173	Proteobacteria	*Desulfovibrionaceae*		0.00 (0.00, 0.14)	0.00 (0.00, 0.16)	0.03 (0.00, 0.09)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	1.0000	0.1538	0.0256	1.0000
OTU0938	Proteobacteria			0.00 (0.00, 0.10)	0.03 (0.00, 0.13)	0.09 (0.02, 0.26)	0.00 (0.00, 0.11)	0.05 (0.00, 0.12)	0.8825	0.1308	0.0044	0.2190
OTU0105	Proteobacteria	*Helicobacteraceae*	*Helicobacter*	2.12 (0.98, 4.74)	0.87 (0.39, 2.27)	0.84 (0.22, 2.82)	1.17 (0.35, 2.29)	0.92 (0.24, 2.28)	0.0148	0.9551	0.5054	0.5054
OTU0579	Firmicutes	*Erysipelotrichaceae*	*Allobaculum*	0.00 (0.00, 0.16)	0.03 (0.00, 0.39)	0.01 (0.00, 0.61)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	1.0000	0.0513	0.0769	1.0000
OTU0958	Firmicutes	*Streptococcaceae*	*Streptococcus*	0.00 (0.00, 0.06)	0.00 (0.00, 0.04)	0.01 (0.00, 0.06)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	0.2000	0.4000	0.0769	1.0000
OTU0647	Firmicutes	*Lactobacillaceae*	*Lactobacillus*	0.00 (0.00, 0.08)	0.09 (0.00, 0.32)	0.00 (0.00, 0.44)	0.00 (0.00, 0.05)	0.00 (0.00, 0.10)	1.0000	0.0087	1.0000	0.9282
OTU0195	Firmicutes	*Lactobacillaceae*	*Lactobacillus*	2.95 (0.60, 7.46)	0.53 (0.15, 1.51)	3.31 (1.37, 4.82)	0.00 (0.00, 0.00)	0.00 (0.00, 0.03)	0.0002	0.0003	0.0002	0.4667
OTU1276	Firmicutes	Unclassified_*Clostridiales*		0.00 (0.00, 0.06)	0.00 (0.00, 0.00)	0.03 (0.00, 0.07)	0.00 (0.00, 0.04)	0.00 (0.00, 0.00)	1.0000	1.0000	0.0629	1.0000
OTU1852	Firmicutes	Unclassified_*Clostridiales*		0.00 (0.00, 0.00)	0.00 (0.00, 0.03)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	1.0000	0.1538	1.0000	1.0000
OTU0277	Bacteroidetes	*Ruminococcaceae*	*Acetivibrio*	0.01 (0.00, 0.07)	0.00 (0.00, 0.03)	0.04 (0.00, 0.15)	0.00 (0.00, 0.08)	0.00 (0.00, 0.03)	0.4126	0.8205	0.0623	1.0000
OTU0274	Firmicutes	*Ruminococcaceae*	*Acetivibrio*	0.01 (0.00, 0.23)	0.00 (0.00, 0.00)	0.00 (0.00, 0.08)	0.00 (0.00, 0.00)	0.00 (0.00, 0.02)	0.0769	1.0000	0.2000	1.0000
OTU0275	Firmicutes	*Ruminococcaceae*		0.12 (0.05, 0.60)	0.10 (0.00, 0.26)	0.05 (0.00, 0.33)	0.01 (0.00, 0.11)	0.05 (0.00, 0.10)	0.0042	0.1141	0.2258	0.7085
OTU2404	Firmicutes	*Ruminococcaceae*	*Oscillibacter*	0.00 (0.00, 0.00)	0.00 (0.00, 0.08)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	0.00 (0.00, 0.03)	1.0000	0.1538	1.0000	1.0000
OTU0178	Firmicutes	*Lachnospiraceae*	*Clostridium* XlVa	0.03 (0.00, 0.09)	0.00 (0.00, 0.12)	0.04 (0.00, 0.17)	0.00 (0.00, 0.00)	0.00 (0.00, 0.03)	0.0070	0.4000	0.0256	0.4667
OTU0580	Firmicutes	*Lachnospiraceae*		0.02 (0.00, 0.22)	0.10 (0.00, 0.42)	0.08 (0.00, 0.33)	0.00 (0.00, 0.03)	0.00 (0.00, 0.08)	0.2821	0.0056	0.0884	0.5692
OTU0374	Firmicutes	*Lachnospiraceae*		0.31 (0.03, 0.81)	0.09 (0.00, 0.49)	0.00 (0.00, 1.03)	0.00 (0.00, 0.35)	0.04 (0.00, 0.62)	0.0019	0.3571	0.4256	0.4779
OTU1643	Firmicutes	*Lachnospiraceae*		0.00 (0.00, 0.00)	0.03 (0.00, 0.64)	0.03 (0.00, 0.10)	0.00 (0.00, 0.05)	0.00 (0.00, 0.03)	1.0000	0.1259	0.0064	1.0000
OTU0215	Firmicutes	*Lachnospiraceae*		2.66 (0.54, 4.35)	1.01 (0.06, 2.78)	0.15 (0.00, 2.05)	0.41 (0.07, 1.07)	0.71 (0.00, 5.50)	0.0047	0.0939	0.2786	0.6454
OTU0328	Firmicutes	*Lachnospiraceae*		0.05 (0.00, 0.23)	0.00 (0.00, 0.08)	0.06 (0.00, 0.10)	0.00 (0.00, 0.13)	0.02 (0.00, 0.06)	0.0200	0.9902	0.1134	0.5301
OTU0419	Firmicutes	*Lachnospiraceae*	*Butyrivibrio*	0.00 (0.00, 0.00)	0.00 (0.00, 0.13)	0.00 (0.00, 0.05)	0.00 (0.00, 0.00)	0.00 (0.00, 0.17)	1.0000	0.1538	0.4667	0.4667
OTU0489	Firmicutes	*Lachnospiraceae*		0.15 (0.08, 0.32)	0.09 (0.00, 0.36)	0.10 (0.03, 0.47)	0.00 (0.00, 0.10)	0.00 (0.00, 0.12)	0.0006	0.0911	0.0135	0.9282
OTU1468	Firmicutes	*Lachnospiraceae*		0.00 (0.00, 0.03)	0.00 (0.00, 0.11)	0.02 (0.00, 0.26)	0.00 (0.00, 0.03)	0.00 (0.00, 0.00)	0.4667	0.9333	0.0816	1.0000
OTU0192	Firmicutes	*Lachnospiraceae*		0.00 (0.00, 0.14)	0.00 (0.00, 0.19)	0.01 (0.00, 0.14)	0.00 (0.00, 0.00)	0.00 (0.00, 0.23)	0.2000	0.1538	0.0769	0.2000
OTU0106	Bacteroidetes	*Rikenellaceae*	*Alistipes*	0.00 (0.00, 0.10)	0.00 (0.00, 0.13)	0.00 (0.00, 0.00)	0.01 (0.00, 0.11)	0.00 (0.00, 0.00)	0.6084	0.7552	0.0769	0.0769
OTU0069	Proteobacteria	*Desulfovibrionaceae*		5.95 (2.90, 15.18)	4.18 (2.05, 5.67)	5.18 (3.66, 8.15)	6.30 (2.71, 10.79)	3.49 (2.13, 7.26)	0.7984	0.0721	0.2345	0.0499
OTU0996	Firmicutes	*Clostridiales_Incertae Sedis XIII*	*Anaerovorax*	0.04 (0.00, 0.14)	0.00 (0.00, 0.03)	0.00 (0.00, 0.03)	0.03 (0.00, 0.13)	0.00 (0.00, 0.05)	0.7030	0.0765	0.0493	0.0797
OTU0947	Firmicutes	*Ruminococcaceae*	*Anaerotruncus*	0.00 (0.00, 0.06)	0.09 (0.00, 0.49)	0.03 (0.00, 0.60)	0.11 (0.02, 0.16)	0.00 (0.00, 0.27)	0.0135	0.9259	0.4859	0.0154

OTU0051	Firmicutes	*Ruminococcaceae*	*Oscillibacter*	0.91 (0.42, 1.99)	1.27 (0.95, 5.49)	2.15 (1.37, 4.33)	3.55 (1.58, 4.13)	0.84 (0.35, 2.40)	0.0003	0.1520	0.2345	0.0006
OTU0092	Firmicutes	*Ruminococcaceae*	*Oscillibacter*	0.10 (0.00, 0.32)	0.25 (0.03, 0.53)	0.07 (0.00, 0.28)	0.25 (0.09, 0.84)	0.05 (0.00, 0.10)	0.0640	0.6943	0.0070	0.0003
OTU0482	Firmicutes	*Ruminococcaceae*	*Flavonifractor*	0.09 (0.00, 0.23)	0.08 (0.00, 0.45)	0.20 (0.00, 0.63)	0.22 (0.07, 0.41)	0.02 (0.00, 0.31)	0.0373	0.2204	0.5737	0.0185
OTU0206	Firmicutes	*Lachnospiraceae*	*Roseburia*	0.14 (0.00, 0.80)	0.19 (0.16, 1.30)	0.03 (0.00, 1.07)	0.51 (0.14, 1.06)	0.14 (0.00, 0.28)	0.2325	0.5358	0.0103	0.0103
OTU0095	Firmicutes	*Lachnospiraceae*	*Lachnospiracea_incertae_sedis*	1.20 (0.00, 5.04)	2.51 (0.62, 4.81)	4.12 (0.26, 11.19)	6.10 (4.20, 13.17)	0.05 (0.00, 3.71)	0.0070	0.0012	0.2786	0.0002
OTU0107	Firmicutes	*Lachnospiraceae*	*Lachnospiracea_incertae_sedis*	0.00 (0.00, 0.24)	0.03 (0.00, 0.19)	0.24 (0.00, 0.37)	0.18 (0.00, 1.21)	0.01 (0.00, 0.57)	0.0483	0.0768	0.6681	0.0348
OTU0018	Firmicutes	*Lachnospiraceae*	*Lachnospiracea_incertae_sedis*	0.09 (0.00, 0.51)	0.05 (0.00, 0.42)	0.04 (0.00, 0.32)	0.14 (0.02, 0.45)	0.02 (0.00, 0.13)	0.3667	0.1501	0.0822	0.0146
OTU0149	Firmicutes	*Lachnospiraceae*	*Marvinbryantia*	0.09 (0.00, 0.69)	0.10 (0.00, 0.26)	0.08 (0.00, 1.34)	0.30 (0.16, 0.98)	0.11 (0.00, 0.65)	0.0278	0.0059	0.1009	0.0494
OTU0193	Firmicutes	*Lachnospiraceae*		0.00 (0.00, 0.03)	0.41 (0.03, 1.01)	0.19 (0.00, 2.97)	0.33 (0.12, 1.28)	0.00 (0.00, 0.02)	0.0002	1.0000	0.1540	0.0002
OTU0284	Firmicutes	*Lachnospiraceae*	*Clostridium* XIVb	0.43 (0.23, 0.88)	0.25 (0.09, 0.83)	0.62 (0.37, 1.22)	0.77 (0.49, 1.20)	0.19 (0.06, 0.73)	0.0148	0.0289	0.1949	0.0011
OTU0604	Firmicutes	*Lachnospiraceae*		0.03 (0.00, 0.11)	0.03 (0.00, 0.05)	0.04 (0.00, 0.17)	0.13 (0.07, 0.41)	0.05 (0.00, 0.10)	0.0011	0.0003	0.0068	0.0019
OTU0075	Firmicutes	*Lachnospiraceae*	*Dorea*	0.91 (0.46, 1.85)	0.50 (0.24, 1.93)	1.34 (1.07, 2.14)	1.49 (0.53, 2.47)	0.65 (0.38, 1.11)	0.1605	0.0289	0.9591	0.0379
OTU0241	Firmicutes	*Lachnospiraceae*	*Dorea*	0.30 (0.00, 1.65)	0.42 (0.00, 0.97)	0.00 (0.00, 1.11)	0.54 (0.06, 1.79)	0.10 (0.00, 1.10)	0.2786	0.4634	0.0193	0.0482
OTU0146	Firmicutes	*Lachnospiraceae*		0.04 (0.00, 0.83)	0.03 (0.00, 0.47)	0.18 (0.00, 0.42)	0.19 (0.08, 0.45)	0.10 (0.00, 0.27)	0.2325	0.0202	0.4396	0.1293
OTU0042	Firmicutes	*Lachnospiraceae*		0.00 (0.00, 0.03)	0.00 (0.00, 0.04)	0.00 (0.00, 0.08)	0.06 (0.00, 0.13)	0.00 (0.00, 0.00)	0.0256	0.0721	0.0928	0.0256
OTU0177	Firmicutes	*Lachnospiraceae*		0.07 (0.00, 0.63)	1.39 (0.98, 2.29)	1.27 (0.05, 3.10)	1.91 (0.66, 3.01)	0.02 (0.00, 1.56)	0.0002	0.3969	0.5054	0.0011
OTU0365	Firmicutes	*Lachnospiraceae*	*Lachnospiracea_incertae_sedis*	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	0.01 (0.00, 0.03)	0.00 (0.00, 0.00)	0.0769	0.1026	0.0769	0.0769
OTU0026	Verrucomicrobia	*Verrucomicrobiaceae*	*Akkermansia*	0.52 (0.02, 2.96)	0.00 (0.00, 1.38)	0.56 (0.00, 4.07)	0.72 (0.00, 12.89)	1.43 (0.56, 8.79)	0.5737	0.0662	0.5949	0.1049
OTU0108	Bacteroidetes	*Porphyromonadaceae*		0.06 (0.00, 0.66)	0.00 (0.00, 0.00)	0.20 (0.00, 0.87)	0.48 (0.23, 3.12)	0.15 (0.00, 4.75)	0.0070	0.0003	0.0368	0.2771
OTU0441	Firmicutes	*Erysipelotrichaceae*	*Allobaculum*	0.00 (0.00, 0.00)	0.13 (0.00, 0.61)	0.00 (0.00, 1.16)	0.01 (0.00, 0.31)	0.00 (0.00, 0.04)	0.0769	0.0497	0.9608	0.1282
OTU0721	Firmicutes	*Erysipelotrichaceae*	*Erysipelotrichaceae_incertae_sedis*	0.01 (0.00, 0.12)	0.00 (0.00, 0.03)	0.00 (0.00, 0.07)	0.02 (0.00, 0.09)	0.01 (0.00, 0.05)	0.8749	0.0895	0.0816	0.4822
OTU0296	Firmicutes	Unclassified_*Clostridiales*		0.49 (0.13, 1.72)	0.12 (0.00, 2.33)	0.44 (0.05, 1.97)	0.44 (0.26, 1.65)	0.53 (0.08, 0.90)	1.0000	0.0199	0.6454	0.7984
OTU0942	Firmicutes	Unclassified_*Clostridiales*		0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	0.01 (0.00, 0.25)	0.00 (0.00, 0.03)	0.0769	0.1026	0.0769	0.1282
OTU1264	Firmicutes	*Ruminococcaceae*		0.00 (0.00, 0.00)	0.00 (0.00, 0.03)	0.00 (0.00, 0.02)	0.04 (0.00, 0.38)	0.00 (0.00, 0.17)	0.0256	0.0373	0.0256	0.2821
OTU0531	Firmicutes	*Ruminococcaceae*	*Clostridium* IV	0.01 (0.00, 0.16)	0.00 (0.00, 0.15)	0.19 (0.09, 0.33)	0.20 (0.00, 0.33)	0.07 (0.02, 0.20)	0.0135	0.0186	0.8785	0.1304
OTU0581	Firmicutes	*Ruminococcaceae*	*Pseudoflavonifractor*	0.02 (0.00, 0.18)	0.13 (0.00, 0.50)	0.12 (0.02, 0.63)	0.26 (0.00, 0.53)	0.21 (0.00, 0.79)	0.0295	0.2785	0.7984	0.6681
OTU0062	Firmicutes	*Ruminococcaceae*	*Pseudoflavonifractor*	0.47 (0.20, 0.85)	0.88 (0.41, 2.06)	0.69 (0.45, 2.48)	0.75 (0.43, 1.06)	0.65 (0.23, 1.40)	0.0650	0.5358	0.9591	0.8785
OTU0029	Firmicutes	*Lachnospiraceae*		0.06 (0.00, 0.46)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	0.02 (0.00, 0.12)	0.15 (0.00, 0.39)	0.2706	0.0373	0.0256	0.0611
OTU0191	Firmicutes	*Lachnospiraceae*		0.09 (0.00, 0.63)	0.13 (0.00, 0.24)	0.00 (0.00, 0.42)	0.07 (0.00, 0.48)	0.05 (0.00, 0.44)	0.8922	0.2925	0.0884	0.9854
OTU0628	Firmicutes	*Lachnospiraceae*	*Clostridium* XlVa	0.00 (0.00, 0.23)	0.00 (0.00, 0.04)	0.00 (0.00, 0.00)	0.03 (0.00, 0.24)	0.00 (0.00, 0.05)	0.5671	0.2200	0.0256	0.3756

OTU0342	**Firmicutes**	***Lachnospiraceae***	***Moryella***	**0.04 (0.00, 0.23)**	**0.00 (0.00, 0.05)**	**0.01 (0.00, 0.19)**	**0.03 (0.00, 0.08)**	**0.01 (0.00, 0.23)**	**0.8757**	**0.0373**	**0.4182**	**0.6892**

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Supplementary Figures

Figure S1

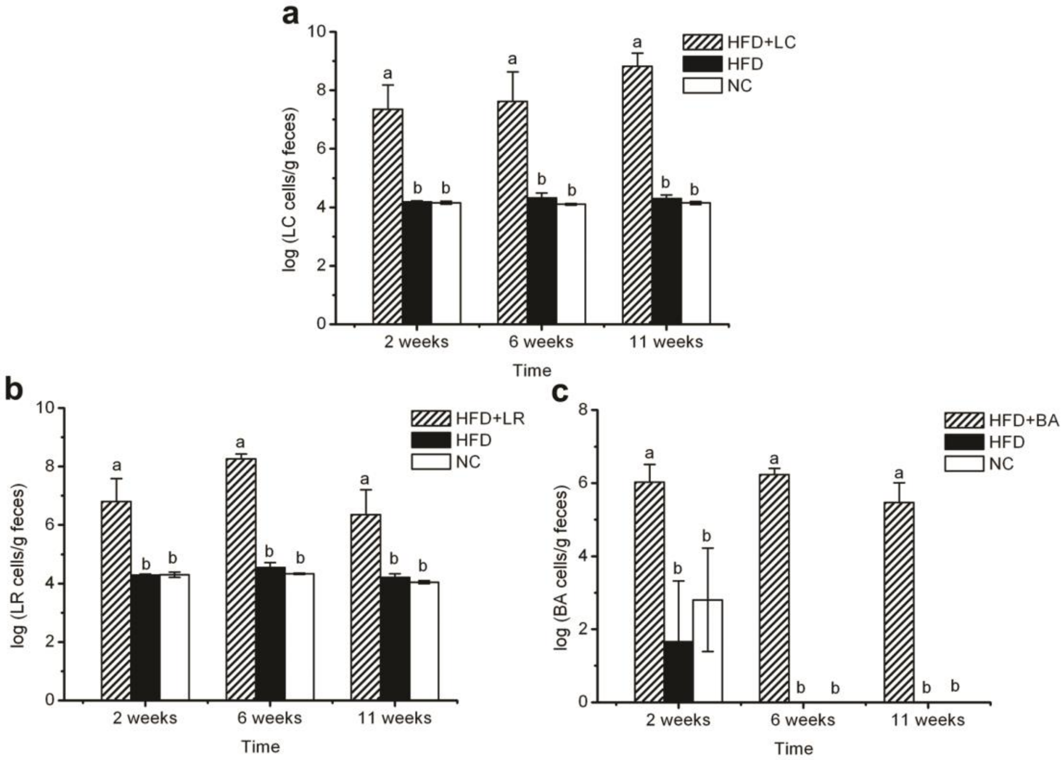

Figure S1 The three candidate probiotics survived in the gut. (a-c) The amount of LC, LR and BA in the feces of mice at 2nd, 6th and 11th week during the probiotic administration quantified by RT-qPCR. Data are shown as means ± SEM. Values of each animal group with same letters are not significantly different by ANOVA followed by Tukey post hoc test. n = 3 mice per group.

Figure S2

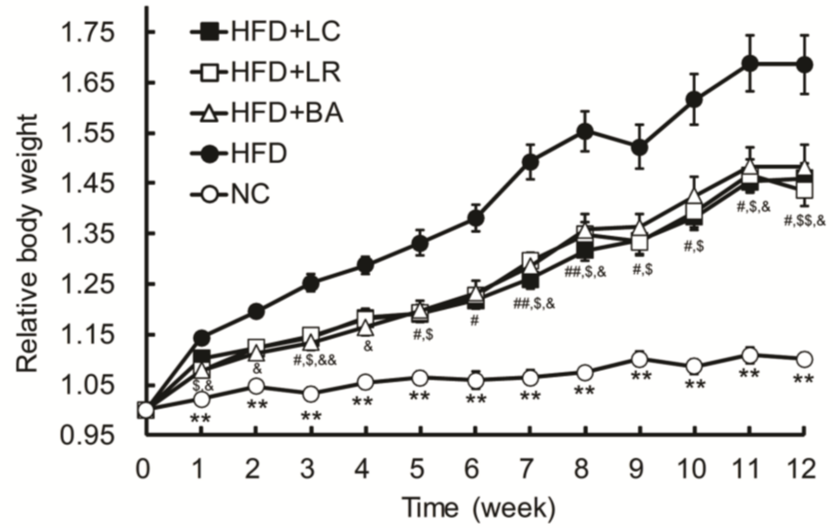

Figure S2 The relative body weight curve of five groups of mice during the 12-week-intervention. Relative body weight was calculated as body weight as percentage of baseline weight for each mouse. Data are shown as means ± SEM. *P < 0.05, **P < 0.01: NC group vs. HFD group; $^{\#}$P < 0.05, $^{\#\#}$P < 0.01: HFD+LC group vs. HFD group; $^{\$}$P < 0.05, $^{\$\$}$P < 0.01: HFD+LR group vs. HFD group; $^{\&}$P < 0.05, $^{\&\&}$P < 0.01: HFD+BA group vs. HFD group by ANOVA followed by Tukey post hoc test. n = 8 mice per group.

Figure S3

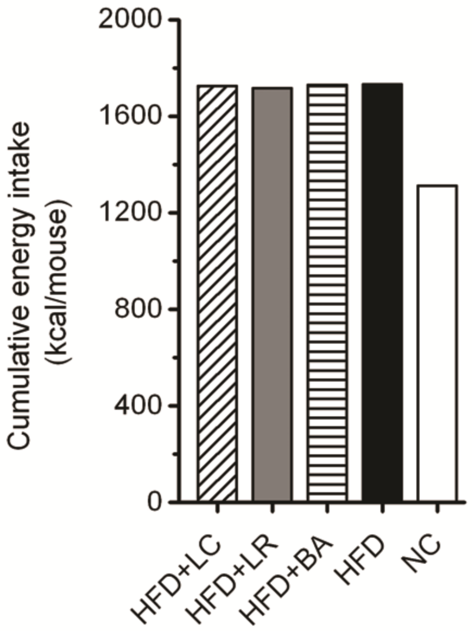

Figure S3 Probiotics did not reduce energy intake. Data are shown as means of food intake of two cages of 8 animals of each animal group.

Figure S4

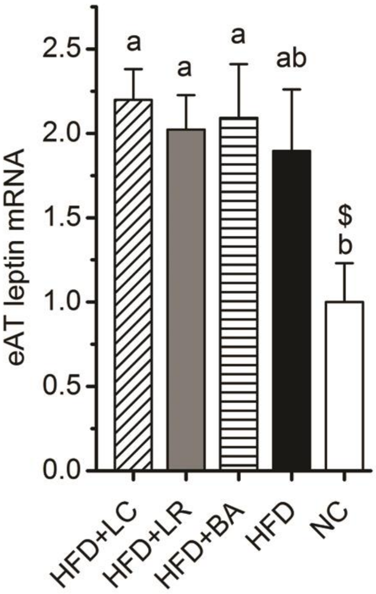

Figure S4 Probiotics did not reduce the expression of leptin gene in eAT. Data are shown as means ± SEM. Values of each animal group with same letters are not significantly different by ANOVA followed by Tukey post hoc test. $: P = 0.057 vs. HFD group. n = 8 mice per group.

Figure S5

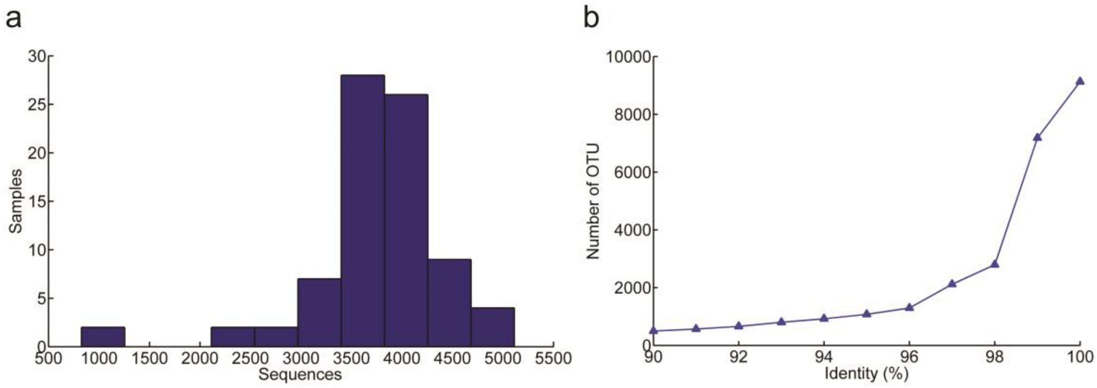

Figure S5 454 pyrosequencing data of 80 fecal samples of five animal groups (8 animals/group) at baseline and 12th week. a: Sample distribution of 301, 568 usable reads, b: The numbers of OTUs identified at several different similarity levels.

Figure S6

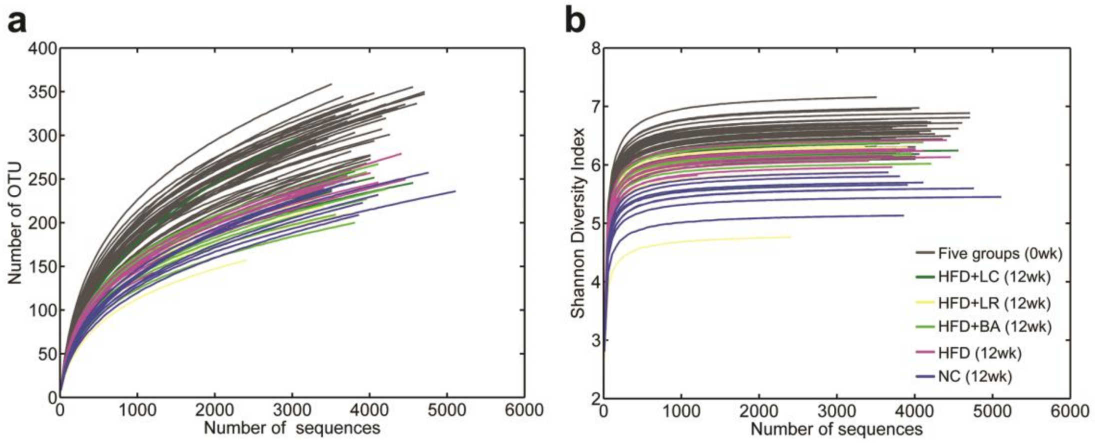

Figure S6 Alpha-diversity analysis of 454 pyrosequencing of 80 fecal samples. (a) Rarefaction analysis. (b) Shannon Diversity Index curves. 0wk: before probiotics intervention, 12wk: after 12 weeks of probiotics intervention.

Figure S7

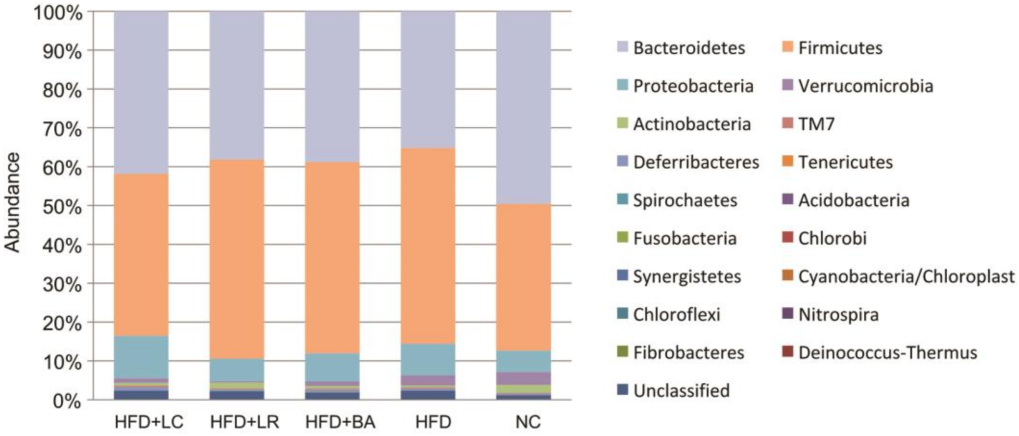

Figure S7 Relative abundance of different phyla in the gut microbiota of five animal groups at 12th week of the trial.

Figure S8

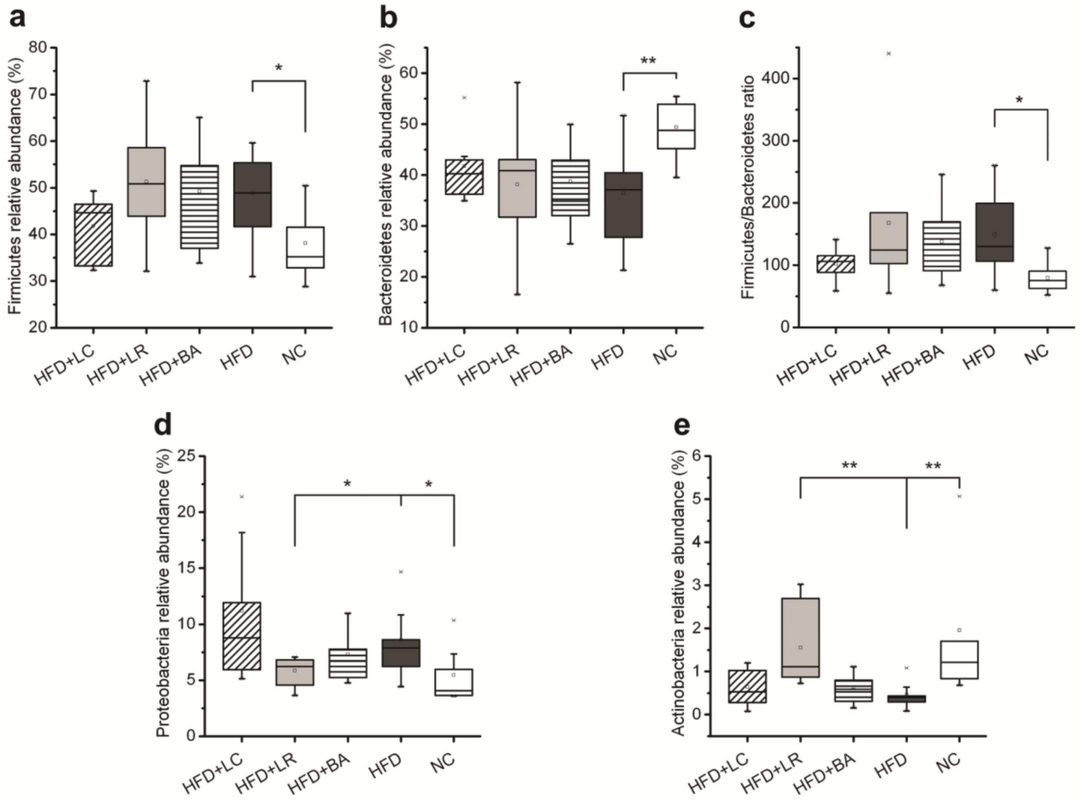

Figure S8 Relative abundance of four predominant phyla which was different between HFD group and the other four groups at 12th week of the trial. (a) Firmicutes, (b) Bacteroidetes, (c) Firmicutes / Bacteroidetes ratio, (d) Proteobacteria, (e) Actinobacteria. In the box plot, the bottom and top are respectively the 25th and 75th percentile, a line within the box marks the median, and a circle in the box shows the mean. Whiskers above and below the box indicate1.5 interquartile range of the lower and upper quartile, and samples beyond are regarded as outliers. *P < 0.05, **P < 0.01 by Mann-Whitney test.

Figure S9

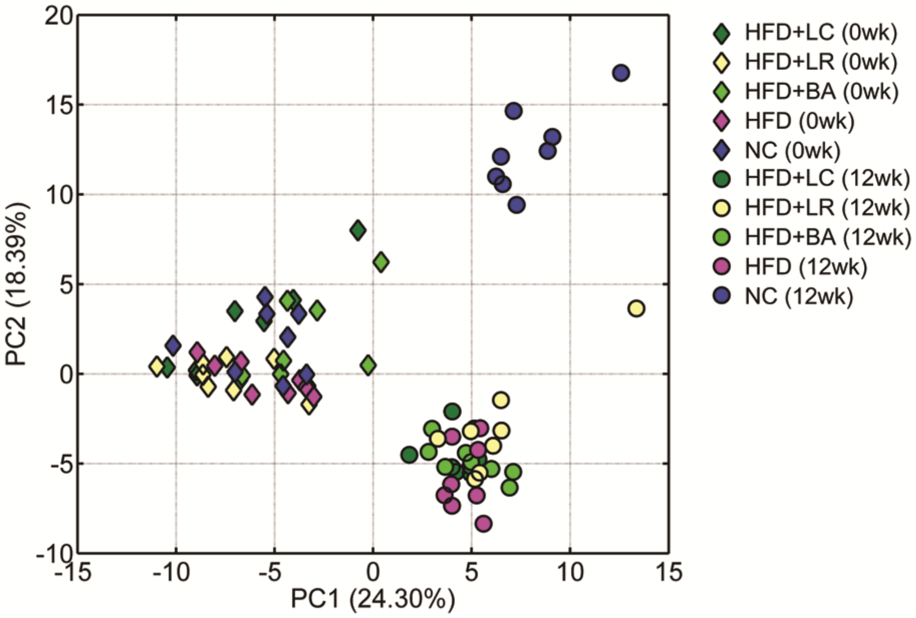

Figure S9 PCA scores plot calculated with the OTU abundance matrix of all animals at baseline and 12th week. Each point represents the microbiota of a mouse. 0wk: before probiotic intervention, 12wk: after 12 weeks of probiotics intervention.

Figure S10

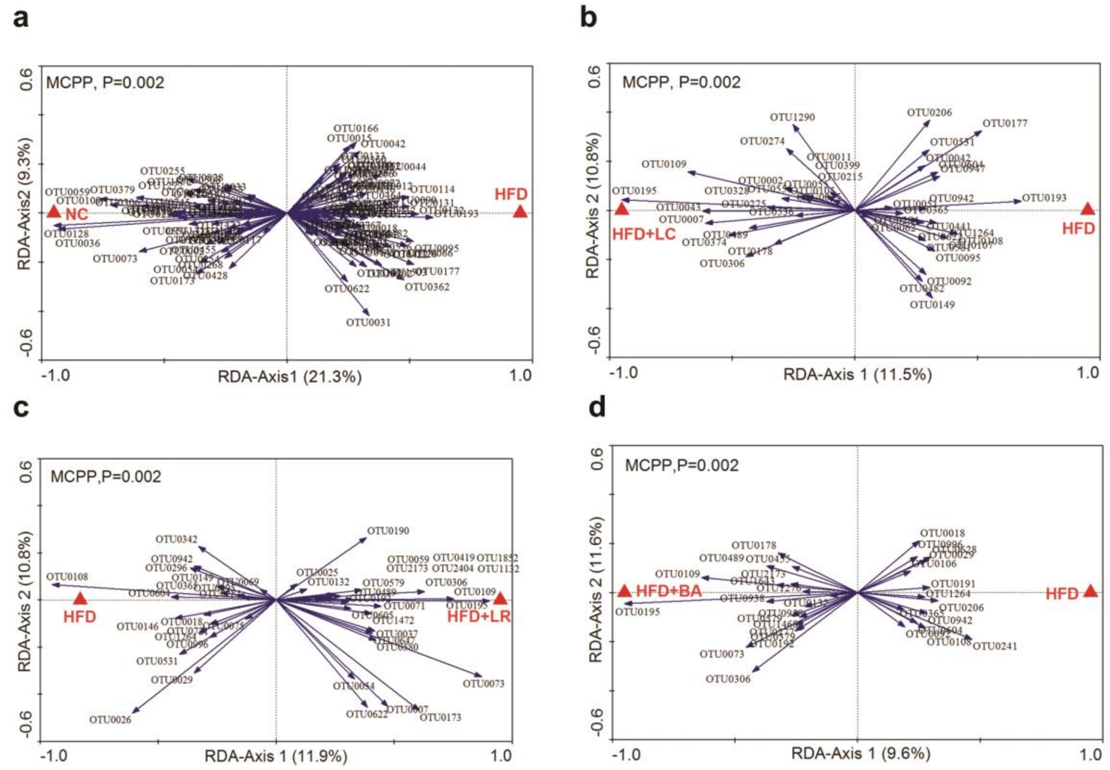

Figure S10 Biplot of the RDA between HFD and NC (a), HFD+LC (b), HFD+LR (c), and HFD+BA (d), respectively on relative abundance of OTUs (Log 10 transformed). Constrained explanatory variables are indicated by red triangles. OTUs that have more than 24% of the variability in their values explained by the canonical axis are indicated by blue arrows. Upper left shows P-value of Monte Carlo Permutation Test.

Figure S11

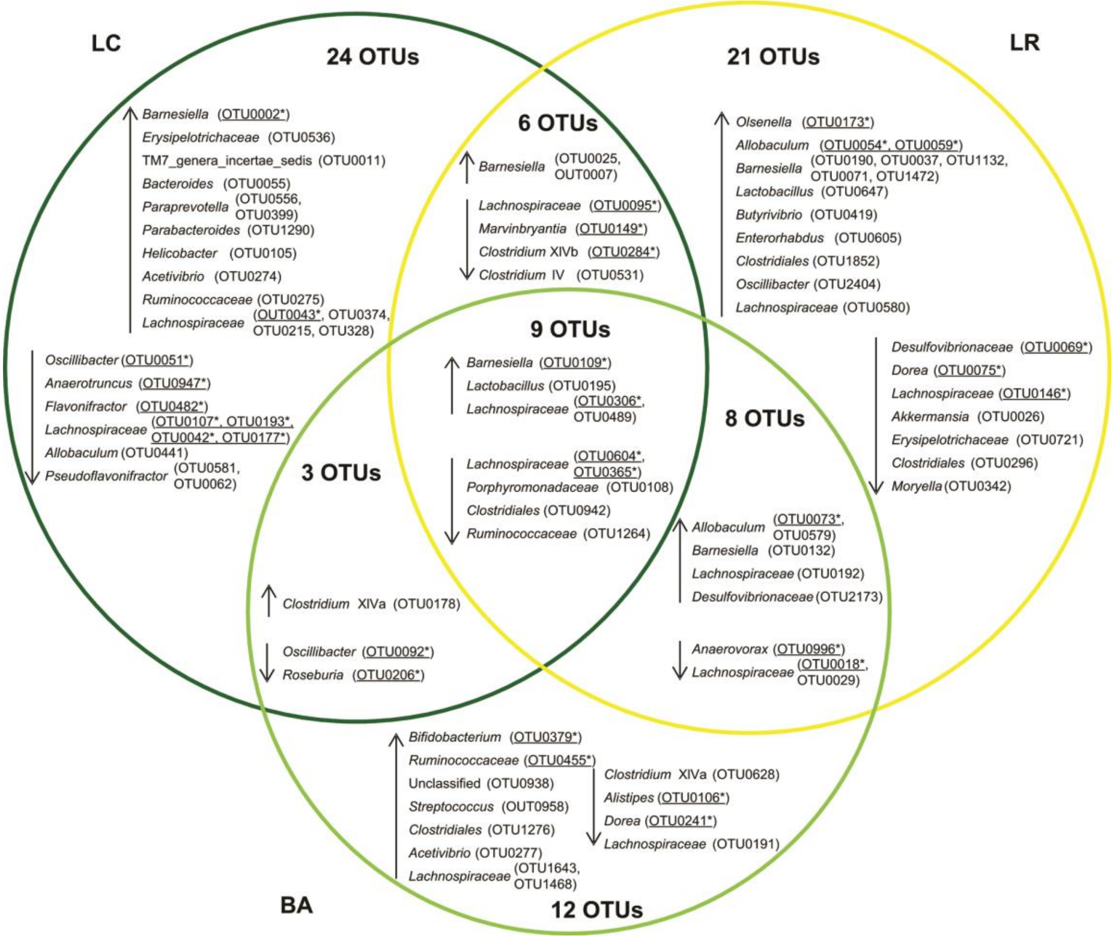

Figure S11 Venn diagrams of 83 OTUs modulated by the three probiotics LC, LR and BA. The OTUs' phylogeny are listed. ↑ means increased by probiotics, and ↓ represents decreased by probotics. * represents the OTU whose abundance was changed by HFD and then the change was reversed by probiotics.