Molecular characterization of gut microbiota in high-lipid diet-induced hyperlipidemic rats treated with simvastatin

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Abstract. Hyperlipidemia is a major risk factor for cardiovascular diseases. Simvastatin (SV), a cholesterol-lowering agent, has been widely used in the treatment of hyperlipidemia. Gut microbiota is known to influence drug response, including that to statins. However, the effect of SV on the gut microbiota of hyperlipidemic rats is not fully understood. To investigate the influence of SV on gut microbiota in hyperlipidemic rats, the molecular characterization of gut microbiota and the potential functions of genes involved in the downstream metabolic pathways were analyzed using high-throughput sequencing technology and the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States approach. The results revealed that SV treatment could reduce the gut microbial diversity and drive marked remodeling of the fecal bacterial community composition. At the phylum level, the relative abundance of *Firmicutes* and *Actinobacteria* was decreased following SV therapy, whereas that of *Bacteroidetes* was elevated. At the genus level, the percentage of the genera *Bacteroides*, *Sutterella* and *Phascolarctobacterium* was significantly increased, but that of *Bifidobacterium*, *Ruminococcaceae_NK4A214*, *Ruminococcaceae_UCG-009*, *Intestinimonas* and *Tyzzerella* was significantly decreased. Additionally, functional prediction analysis indicated that in the SV-associated microbiota, genes involved in energy, carbohydrate, amino acid and nucleotide metabolism likely exhibited enrichment. Briefly, to the best of our knowledge, the present study was the first to establish a profound and comprehensive association between the SV-induced alterations of the gut flora and the consequent influences of downstream metabolic pathways by gut microbiota. These findings suggested that the gut microbiota may contribute to the SV hypolipidemic efficacy in the progression of hyperlipidemia, which could provide insights for the prevention and treatment of hyperlipidemia.

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Introduction

Hyperlipidemia, which refers to abnormally elevated levels of lipids, such as cholesterol, and lipid proteins in the blood, increases the risk of arteriosclerosis, coronary heart disease, cerebral stroke and myocardial infarction in humans (1-3). Simvastatin (SV) is a lactone prodrug. It undergoes reversible de-esterification to its active 6-hydroxyl acid and serves as an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase to regulate hepatic cholesterol production (4). Additionally, SV is one of the most commonly prescribed statins, a safe and effective cholesterol-lowering drug for the clinical treatment of hyperlipidemia (5).

Other studies have suggested that metabolites produced by gut bacteria can enter the bloodstream by absorption, enterohepatic circulation or impaired gut barrier function (6,7), which can affect the metabolism of xenobiotics and serves an important role in disease onset and progression (8). On the other hand, the microbiota composition is also the result of the equilibrium between the ability of the host to withstand the selective pressure of the immune system and the ability to take advantage of available nutrients (9). In addition, microbial influences on drug response and bioavailability by their metabolic or peptide products on the host immune system or host metabolism, which is called indirect microbial effects, have attracted much attention (10,11). Although growing evidence has demonstrate that the gut microbiome is involved in variability of drug response and bioavailability, the underlying molecular mechanisms remain largely unknown (12,13). Zimmerman *et al* (13) measured the ability of diverse human gut bacteria to metabolize 271 oral drugs and found ~2/3 of the assayed drugs are metabolized by at least one strain, and 30 microbiome-encoded enzymes are validated. It is, therefore, possible to improve drug efficacy or minimize drug toxicity by manipulating the gut microbiome in the host (12,14).

In a previous study, variations in the therapeutic benefits and low-density lipoprotein cholesterol (LDL-C) reduction of SV therapy have been observed between 'good' and 'poor' responders (15). However, the mechanisms underlying the interindividual variation of cholesterol lowering during SV therapy are poorly understood. Therefore, Trupp *et al* and Kaddurah-Daouk *et al* (16,17) conducted a series of investigations to decipher the association between metabolic changes and efficacy of SV in 'good' and 'poor' responders using the metabolomic approach. They detected that plasma concentrations of SV are positively associated with microbially-synthesized secondary bile acids (18). The evidence highlighted the involvement of gut microbiota in affecting individual response to SV. Another study has demonstrated that one of the most significant differences in active fecal suspension is SV fragmentation (19). The data suggested that the degradation of SV by hydrolytic cleavage of methyl butanoic acid from the SV backbone may be associated with the intestinal microflora. Although statins have been implied to reduce growth and virulence in a number of bacterial pathogens due to their anti-inflammatory and immunomodulatory activities (20-22), the composition and metabolism characteristics of gut microbiota following SV treatment, particularly in a diseased state, are poorly understood. The present study was designed to obtain insights into the gut microbiota response to SV treatment in a hyperlipidemia disease model, as well as the underlying mechanisms of the metabolic pathways involved in gut flora interactions. Gut microbiota in fecal samples from high-lipid diet-fed rats following SV treatment were comprehensively evaluated by 16S rRNA gene high-throughput sequencing and Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) algorithm analysis. The results may help obtain an improved understanding of the associations among gut microbiota, hyperlipidemia and the hypolipidemic efficacy of SV.

Materials and methods

Materials and reagents. SV (20 mg) was purchased from Merck Sharp & Dohme-Hoddesdon. DNA extraction was performed using the fast DNA stool mini kit (Qiagen GmbH). High-throughput sequencing was performed using Phusion High-Fidelity PCR Master mix (New England BioLabs, Inc.), the Qiagen Gel Extraction kit (Qiagen GmbH) and the TruSeq DNA PCR-Free Sample Preparation kit (Illumina, Inc.). Commercial assay kits for total cholesterol (TC), triglycerides (TG), LDL-C and high-density lipoprotein cholesterol (HDL-C) were purchased from Nanjing Jiancheng Bioengineering Institute.

Animal treatment and experimental design. All animal experiments were carried out in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and were approved by the Ethical Committee of Xi'an Jiaotong University School of Medical Sciences (approval no. 2017-288). A total of 24, 8-week-old male Sprague Dawley rats, weighing 180-220 g were purchased from the Experimental Animal Center of Xi'an Jiaotong University (Xi'an, China) and maintained under controlled specific pathogen-free environmental conditions (temperature, 24±2˚C; relative humidity, 55±15%; 12-h light/dark cycle) with *ad libitum* access to food and water.

All 24 rats were acclimatized for 1 week prior to the experiments, and then fed with high-lipid diet enriched with 1% (w/w) cholesterol, 10% (w/w) lard, 5% egg yolk, 0.2% propylthiouracil, 1% sodium tauroglycocholate and 82.8% basic diet (23,24) during the entire study period. Following 4 weeks of high-lipid diet feeding, the levels of TC, TG, LDL-C and HDL-C in rat serum were determined and compared with those of the same rats prior to the experiment. Subsequently, the 24 rats in the hyperlipidemia disease model (24) were randomly divided into three groups (n=8). Two groups were treated with SV at a dose of 10 $mg/kg^{-1}/day^{-1}$ and 40 $mg/kg^{-1}/day^{-1}$ each, as previously described (25-27). The remaining group was considered to be the control group (0 $mg/kg^{-1}/day^{-1}$). The suspensions of SV (10 and 40 mg/kg^{-1}) were prepared in sterile water and administered daily via gastric gavage at approximately the same time, at noon. Fecal samples were collected at the end of weeks 2 and 4 of the SV treatment period. As a consequence, a total of 48 fecal samples were divided into six groups: TW0mg, TW10mg and TW40mg (2-week SV treatment); and FW0mg, FW10mg and FW40mg (4-week SV treatment). TW0mg and FW0mg were the control groups, and the other four groups were considered to be treatment groups. All fecal samples were stored immediately at -80˚C until DNA extraction for subsequent sequencing.

Bacterial genomic DNA extraction. Each fecal sample (~200 mg) was resuspended in Qiagen InhibitEX buffer (Qiagen GmbH) and thoroughly homogenized for 2 min. Bacterial genomic DNA was extracted from the supernatant using the QIAamp Fast DNA stool Mini kit (Qiagen GmbH), according to the manufacturer's protocol. The extracted bacterial genomic DNA was estimated by measuring the absorbance at 260 nm using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.) prior to downstream processing.

16S rRNA gene high-throughput sequencing. For each sample, the extracted bacterial genomic DNA was diluted to a 1 ng/μl working stock, which was used as a template for PCR. The barcoded primers flanked the V3-V4 region of the bacterial 16S rRNA gene. The primer sequences were as follows: 341F, 5'-CCTAYG GGRBGCASCAG-3' and 806R, 5'-GGACTACNNGGGTAT CTAAT-3'. All PCR reactions were carried out using Phusion High-Fidelity PCR Master Mix (New England BioLabs, Inc.). The thermocycling conditions were as follows: Pre-denaturation at 98˚C for 1 min, followed by 30 cycles of denaturation at 98˚C for 10 sec, annealing at 50˚C for 30 sec and extension at 72˚C for 30 sec; final extension at 72˚C for 5 min. Resulting amplicons were confirmed in 2% agarose gel containing ethidium bromide and purified using a Qiagen Gel Extraction kit (Qiagen GmbH). Sequencing libraries were constructed using the TruSeq DNA PCR-Free Sample Preparation kit (Illumina, Inc.), according to the manufacturer's protocol. Pooled amplicons were paired-end sequenced (2x250) on an Illumina HiSeq2500 platform, according to the manufacturer's protocol. The samples were sent to Novogene Co., Ltd. for sequencing analysis.

Data analysis. Paired-end reads were merged using FLASH (version 1.2.7; http://ccb.jhu.edu/software/FLASH/) (28).

Following merging, quality filtering on the raw tags was performed under specific filtering conditions to obtain the high-quality clean tags (29), according to the Quantitative Insights Into Microbial Ecology (QIIME; version 1.7.0) quality-control process (30). Using the UCHIME algorithm, chimera sequences were detected and then removed against the Gold reference database (31,32) and the effective tags were finally obtained. Similar sequences were assigned to operational taxonomic units (OTUs) using the threshold of 97% identity (UPARSE version 7.0.1001; http://drive5.com/uparse/). Each representative sequence was matched against the SSUrRNA database of SILVA using Mothur (version 1.39.3) to annotate taxonomic information (33,34). OTUs abundance information was normalized using a standard of sequence number corresponding to the sample with the least sequences.

Rarefaction and rank-abundance curves were drawn using the R software (version 2.15.3; https://www.r-project.org/). α diversity analysis including Chao1 index, abundance-based coverage estimator (ACE) index, Shannon index and Simpson index were estimated using QIIME software. Phylogenetic β diversity distances, including unweighted and weighted UniFrac distances, were calculated using QIIME software. Principal coordinates analysis (PCoA) was performed to visualize the similarities or dissimilarities of variables from complex multidimensional data. This analysis was visualized using the WGCNA package (version 1.51; https://cran.r-project.org/web/packages/WGCNA/), stats package (version 3.4.0; https://www.rdocumentation.org/packages/stats/versions/3.4.0) and the ggplot2 package (version 2.2.0; https://cran.r-project.org/web/packages/ggplot2/) in the R software. Unweighted pair-group method with arithmetic means (UPGMA) clustering was performed using QIIME software as a type of hierarchical clustering to interpret the distance matrix using average linkage. The significant differences of the bacterial community structure were tested by analysis of similarities (ANOSIM) and permutational multivariate analysis of variance using distance matrices (ADONIS) in the R software with the use of the vegan package (version 2.4-0; https://cran.r-project.org/web/packages/vegan/). Bacterial taxa with different abundances among different groups were detected using MetaStat analysis (35), and only taxa with mean relative abundance of >0.1% were considered. P-values were corrected for multiple comparisons with Benjamini and Hochberg false-discovery rate correction (q-value) (36).

The associations among microbial taxa could be captured by co-occurrence networks. Genera from 16S rRNA gene sequencing were used to calculate Spearman's correlation coefficient for the TW0mg, TW10mg and TW40mg groups (37,38).

Functional annotation and profiling. The 16S rRNA gene study is a common method of identifying the bacterial taxonomic composition of fecal samples, but it has disadvantages in directly identifying the functional capabilities of the bacteria (39). PICRUSt algorithm analysis was used to analyze and predict the functional capacity of the intestinal bacterial community (39). The sequences were processed using QIIME. To make closed-reference picked OTUs compatible with PICRUSt, the Greengenes reference database (version 13.5; ftp://greengenes.microbio.me/greengenes_release/gg_13_5/gg_13_5_otus.tar.gz) was used. Following the filtering and normalizing of the OTU table, functional predictive assignment was performed coordinating with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (40).

Statistical analysis. Data are presented as the mean ± SD (levels of TC, TG, LDL-C and HDL-C; average length and number of sequences) or mean ± standard error of the mean (relative abundance of operational taxonomic units at species level). The levels of TC, TG, LDL-C and HDL-C were compared with those of the same rats before the experiment using a paired-sample t-test. Significant differences between treated and untreated groups (TW10mg vs. TW0mg; TW40mg vs. TW0mg; FW10mg vs. FW0mg; FW40mg vs. FW0mg) in α diversity and functional prediction were tested by Kruskal-Wallis, followed by Dunn's post hoc test. ANOSIM and ADONIS were used to test the existence of different compositions between treated and untreated groups. $P<0.05$ was considered to indicate a statistically significant difference. MetaStat analysis investigated the differentially abundant bacterial taxa between treated and untreated groups, and $P<0.05$ with $q<0.05$ was considered to indicate a statistically significant difference. All statistical analysis was carried out using SPSS (version 22.0; SPSS, Inc.) or R (version 2.15.3) software.

Results

Hyperlipidemia model. After a 4-week administration of a high-lipid diet, the levels of TC, TG, LDL-C and HDL-C (TC, 9.50±3.85 mmol/l; TG, 1.44±0.54 mmol/l; LDL-C, 6.34±2.35 mmol/l; HDL-C, 0.94±0.38 mmol/l; mean ± SD) were compared with those of the same rats before the experiment (data not shown). There were significant increases in the TC and LDL-C levels ($P<0.001$). TG was slightly increased ($P<0.05$), whereas HDL-C was decreased ($P<0.05$). The results clearly demonstrated that the hyperlipidemia model had been successfully established in these 24 Sprague Dawley rats.

Overview of sequencing analysis. A total of 4,082,278 raw sequences were generated in the present study (Table SI). Following sequence trimming, quality filtering and the removal of chimeras, 2,802,025 high-quality sequences remained, with an average length of 416±2 bp (mean ± SD). The mean number of sequences per sample was 58,376±4,050 (mean ± SD; data not shown). The rarefaction and rank abundance curves of all samples were stable, indicating that species representation in each sample had approached the plateau phase. This meant that no more bacteria would be detected with additional sequencing efforts (Fig. S1A and B).

These high-quality sequences were assigned to a total of 48,600 OTUs by the UPARSE pipeline based on 97% similarity, with an average of 1,012 OTUs per sample (Table SI). In total, 2,316 OTUs were singletons. A total of 99.98±0.11% (mean ± SD) high-quality sequences were classified to the phylum level, 98.71±0.47% (mean ± SD) to the family level, 71.80±7.84% (mean ± SD) to the genus level and 18.75±9.93% (mean ± SD) to the species level. The results demonstrated that the bacteria belonged to 29 phyla, 66 classes, 128 orders, 232 families, 362 genera and 111 species (data not shown).

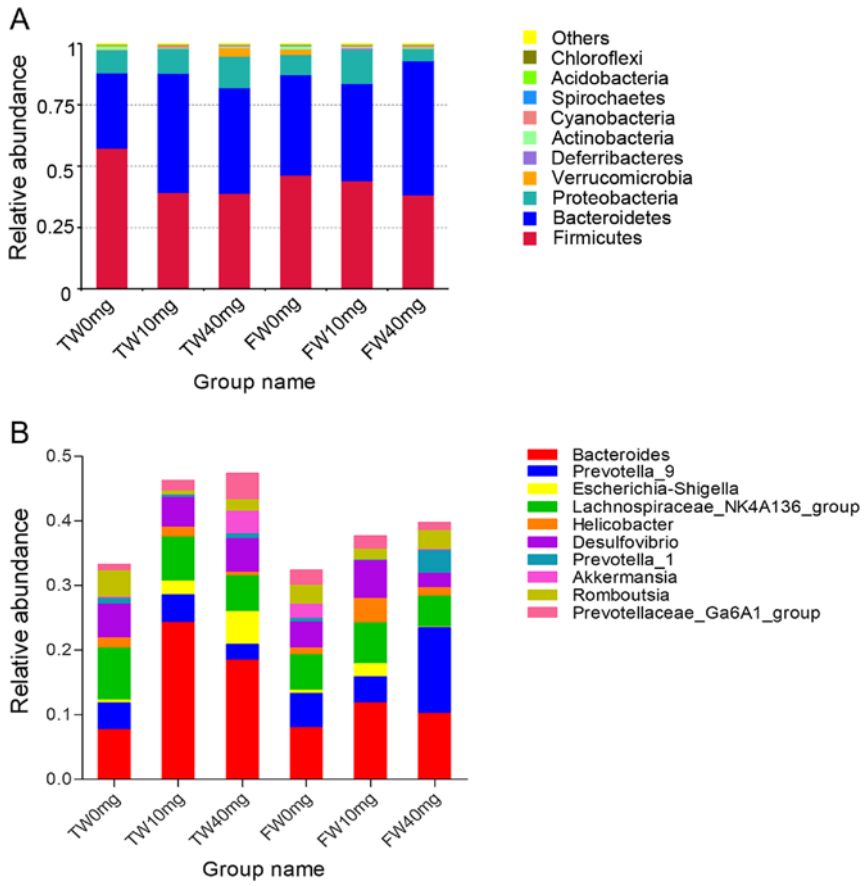

Figure 1. Taxonomic profiles of the fecal bacteria from 16S rRNA gene sequencing. (A) Relative abundances of top 10 phyla of each group. (B) Relative abundances of top 10 genera of each group. TW, 2-week SV treatment; FW, 4-week SV treatment.

Relative abundance of gut bacteria in different groups. The top 10 phyla and top 10 genera in gut bacteria relative abundance are shown in Fig. 1. *Firmicutes* (38.22-57.39%) and *Bacteroidetes* (30.64-54.80%) were the most prevalent phyla in all groups, followed by *Proteobacteria* and *Verrucomicrobia*. These phyla accounted for ~97.53, 98.04, 98.30, 97.76, 97.52 and 98.07% of the reads in the TW0mg, TW10mg, TW40mg, FW0mg, FW10mg and FW40mg groups, respectively (Table SII). Additionally, the *phylum Actinobacteria* is shown in Table SII. Only 0.029, 0.020, 0.021, 0.026, 0.023 and 0.013% of sequences were unclassified in the TW0mg, TW10mg, TW40mg, FW0mg, FW10mg and FW40mg groups, respectively. The relative abundance of *Firmicutes* in the SV treatment groups (TW10mg, 39.27%; TW40mg, 38.88%; FW10mg, 44.07%; FW40mg, 38.22%) was lower than that in the control groups (TW0mg, 57.39%; FW0mg, 46.36%; Fig. 1A; Table SII).

At the genus level, *Bacteroides* (7.70-24.25%) was the most predominant gut bacterial genus in the TW0mg, TW10mg, TW40mg, FW0mg and FW10mg groups; other major genera included *Prevotella_9*, *Lachnospiraceae_NK4A136_group*, *Escherichia-Shigella*, *Helicobacter*, *Desulfovibrio*, *Prevotella_1*, *Akkermansia*, *Romboutsia* and *Prevotellaceae_Ga6A1_group* (Fig. 1B). It was identified that the relative abundance of genus *Bacteroides* in the SV treatment groups (TW10mg, 24.25%; TW40mg, 18.42%; FW10mg, 11.85%; FW40mg, 10.23%) was higher than that in the control groups (TW0mg, 7.70% and FW0mg, 8.02%; Fig. 1B; Table SII).

Characterization of bacterial α diversity. The observed OTUs, Chao1, ACE, Shannon and Simpson indices were calculated for each sample to evaluate the richness, evenness and diversity of the microbial communities (Table SI). More specifically, as compared with the TW0mg group, the values of the OTUs and Shannon indices significantly decreased in the TW10mg and TW40mg groups, whereas the values of the Chao1 and ACE indices significantly decreased in the TW40mg group. Compared with the FW0mg group, the Shannon and Simpson indices were significantly decreased in the FW40mg group ($P<0.05$; Fig. 2A). In addition, Good's coverage was 99.6% on average. This result indicated that the 16S rRNA sequences identified in these groups represented the major bacterial sequences in the samples.

Furthermore, Venn diagrams were used to compare the similarities and differences among the microbial communities in different groups. The TW0mg, TW10mg and TW40mg groups had 1,287 OTUs in common. These OTUs represented 68.35, 74.35 and 80.09% to their respective total OTUs. Additionally, the FW0mg, FW10mg and FW40mg groups had 1,326 OTUs in common. These OTUs represented 74.66, 75.68 and 76.29% to their respective total OTUs (Fig. S1C and D).

Characterization of bacterial β diversity. PCoA was performed to visualize the similarities or dissimilarities of the intestinal microbiota composition among different groups. According to PC1 and PC2 analysis (47.37 and 17.30% of variance explained, respectively), the microbial communities of

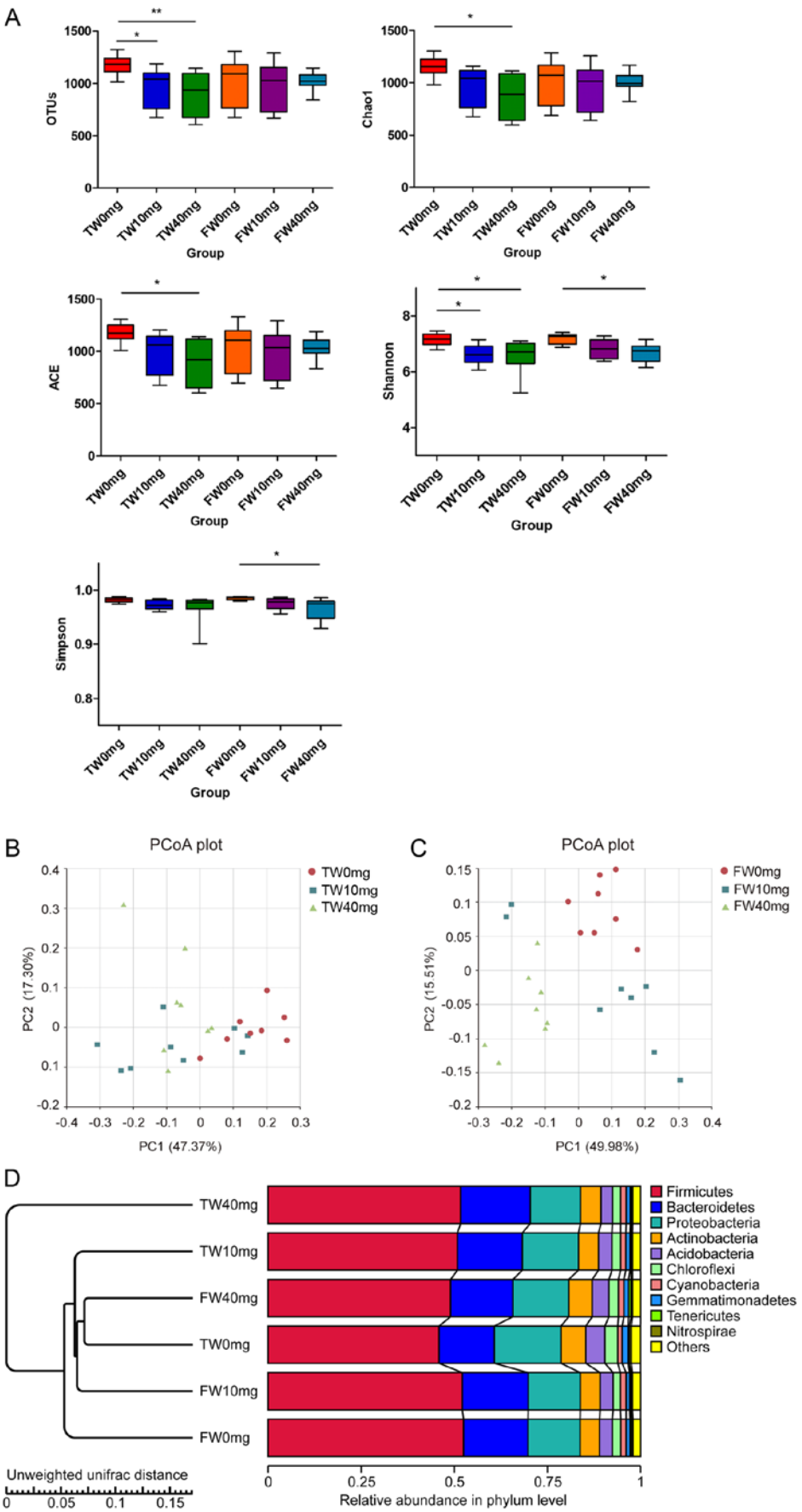

Figure 2. α and β diversity of bacterial community. (A) Boxplots of α diversity indices (observed OTUs, Shannon, Simpson, Chao1 and ACE) in each group. *P<0.05, **P<0.001. Kruskal-Wallis followed by Dunn's post hoc test was used to test significant differences. PCoA plots for samples following SV treatment for (B) 2 weeks and (C) for 4 weeks. Each point corresponds to an individual rat. (D) Unweighted pair-group method with arithmetic means tree, all revealing differences among six groups based on the unweighted Unifrac distances of OTUs community. OTU, Operational Taxonomic Unit; PCoA, principal coordinate analysis; SV, simvastatin; ACE, abundance-based coverage estimator; TW, 2-week SV treatment; FW, 4-week SV treatment.

the TW0mg, TW10mg and TW40mg groups were separated clearly from one another (Fig. 2B). Following 4 weeks of treatment, the microbial communities of the FW0mg, FW10mg and FW40mg groups were grouped into three distinct clusters based on PC1 and PC2 analysis (49.98 and 15.51% of variance explained, respectively; Fig. 2C). The PCoA plots demonstrated the dissimilarities of microbial community structure among these groups. Additionally, this observation was supported by UPGMA analysis based on the unweighted UniFrac distances (Fig. 2D). Furthermore, the results of the UPGMA analysis revealed that the composition of gut microbiota could be altered by SV treatment.

Furthermore, ANOSIM and ADONIS analyses were used to test the differences in bacterial communities between different groups. An R value of >0 was considered well separated by ANOSIM analysis. Higher R^2 values suggested larger intergroup differences, as determined by ADONIS analysis (Fig. S2; Table I). The results of ANOSIM and ADONIS analyses revealed that there were significant differences in the fecal bacterial community between the treatment and control groups (P<0.05).

Table I. ANOSIM and ADONIS analysis of the community structure of fecal samples.

	ANOSIM		ADONIS	
Comparison	R	P-value	R^2	P-value
TW0mg vs. TW10mg	0.8343	0.001	0.36395	0.001
TW0mg vs. TW40mg	0.5642	0.001	0.26047	0.001
FW0mg vs. FW10mg	0.6088	0.001	0.23014	0.001
FW0mg vs. FW40mg	0.6752	0.001	0.25363	0.001

R value was considered well separated if >0. Higher R^2 values suggested a larger intergroup difference. P<0.05 was considered to indicate a statistically significant difference. ANOSIM, analysis of similarities; ADONIS, permutational multivariate analysis of variance using distance matrices; TW, 2-week SV treatment; FW, 4-week SV treatment.

Changes in bacterial communities following SV treatment. At the phylum level, significant differences in the major taxonomical profiles (mean relative abundance of >0.1%) of fecal microbiota among different groups were further identified using MetaStat analysis. The relative abundances of phylum *Firmicutes* and *Actinobacteria* in the fecal microbiota were decreased following SV treatment, whereas that of *Bacteroidetes* was elevated. Specifically, as compared with the TW0mg group, the phylum *Firmicutes* and *Actinobacteria* in the TW10mg and TW40mg groups were significantly decreased (q<0.05). By contrast, there was a significant increase in the relative abundance of the phylum *Bacteroidetes* in the TW10mg, TW40mg and FW40mg groups (TW0mg vs. TW10mg, q<0.05; TW0mg vs. TW40mg, q<0.05; FW0mg vs. FW40mg, q<0.05; Fig. 3A; Table SII).

A total of 16 genera (average relative abundance of >0.1%; P<0.05; q<0.05) exhibiting different relative abundances were identified between the control and treatment groups (Table SII). The relative abundances of the *Bacteroides* and *Phascolarctobacterium* genera showed a significant increase following SV treatment for 2 weeks (TW10mg, TW40mg; q<0.05) compared with that in the TW0mg group (q<0.05), whereas that of *Bifidobacterium* and *Tyzzerella* displayed a significant decrease (q<0.05). In addition, the relative abundance of the genus *Sutterella* was significantly elevated in the TW10mg group compared with that in the TW0mg group (q<0.05); however, the genus *Intestinimonas* was less abundant in the TW10mg group than in the TW0mg group (q<0.05). Furthermore, the relative abundance of genus *Ruminococcaceae_UCG-009* was significantly decreased in the TW40mg compared with in the TW0mg group. The genus *Ruminococcaceae_NK4A214* was also decreased in the FW40mg group compared with in the FW0mg group (Fig. 3B).

A total of 35 sequences were classified to the species level, and certain species exhibited significant differences in relative abundance following SV treatment (Table II). A higher percentage of *Bacteroides_caccae*, *Parabacteroides_distasonis*, *Escherichia_coli*, *Parasutterella_secunda* and *Bacteroides_fragilis* was observed in the TW10mg group, as compared with the TW0mg group. In addition, the relative abundances of *Bacteroides_intestinalis*, *Bacteroides_vulgatus* and *Bacteroides_uniformis* were significantly increased in both the TW10mg and TW40mg groups, whereas *Lachnospiraceae_bacterium_COE1* was less abundant. The percentage of *Clostridiales_bacterium_CIEAF_020* decreased in the TW40mg and FW40mg groups compared with in the TW0mg and FW0mg groups, respectively.

Co-occurrence networks of bacterial genera. Based on the aforementioned analyses, the change of the intestinal microbiota was particularly caused following SV treatment for 2 weeks. The co-occurrence networks mainly focused on TW0mg, TW10mg and TW40mg groups. Based on Spearman's correlation coefficient (r), the co-occurrence networks deduced from the genera were separately constructed for the rats of these three groups (Fig. 4). The results revealed that the predominant genera were from the *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* phyla. Samples from the TW0mg group had a lower network complexity than those from other groups. In addition, genera from *Bacteroidetes* in the TW10mg and TW40mg groups had a lower negative correlation with other genera, as compared with the TW0mg group, but genera from *Firmicutes* and *Proteobacteria* showed a higher positive correlation. Notably, genera from the *Firmicutes* showed an increased positive correlation in the TW10mg and TW40mg groups, mainly including genera within the *Ruminococcaceae*, *Lachnospiraceae* and *Family_XIII* families.

Functional annotation of the gut microbiota following SV treatment. The function of microbiota was investigated based on information from the 16S rRNA gene and OTUs using the PICRUSt algorithm. From the 6,112 predicted KEGG Orthology terms, 328 KEGG pathways were tested. The top 10 abundant categories at level 2 are shown in Fig. 5A. The third level of the KEGG pathway is shown in a heatmap (Fig. 5B). The SV-associated microbial genes involved in

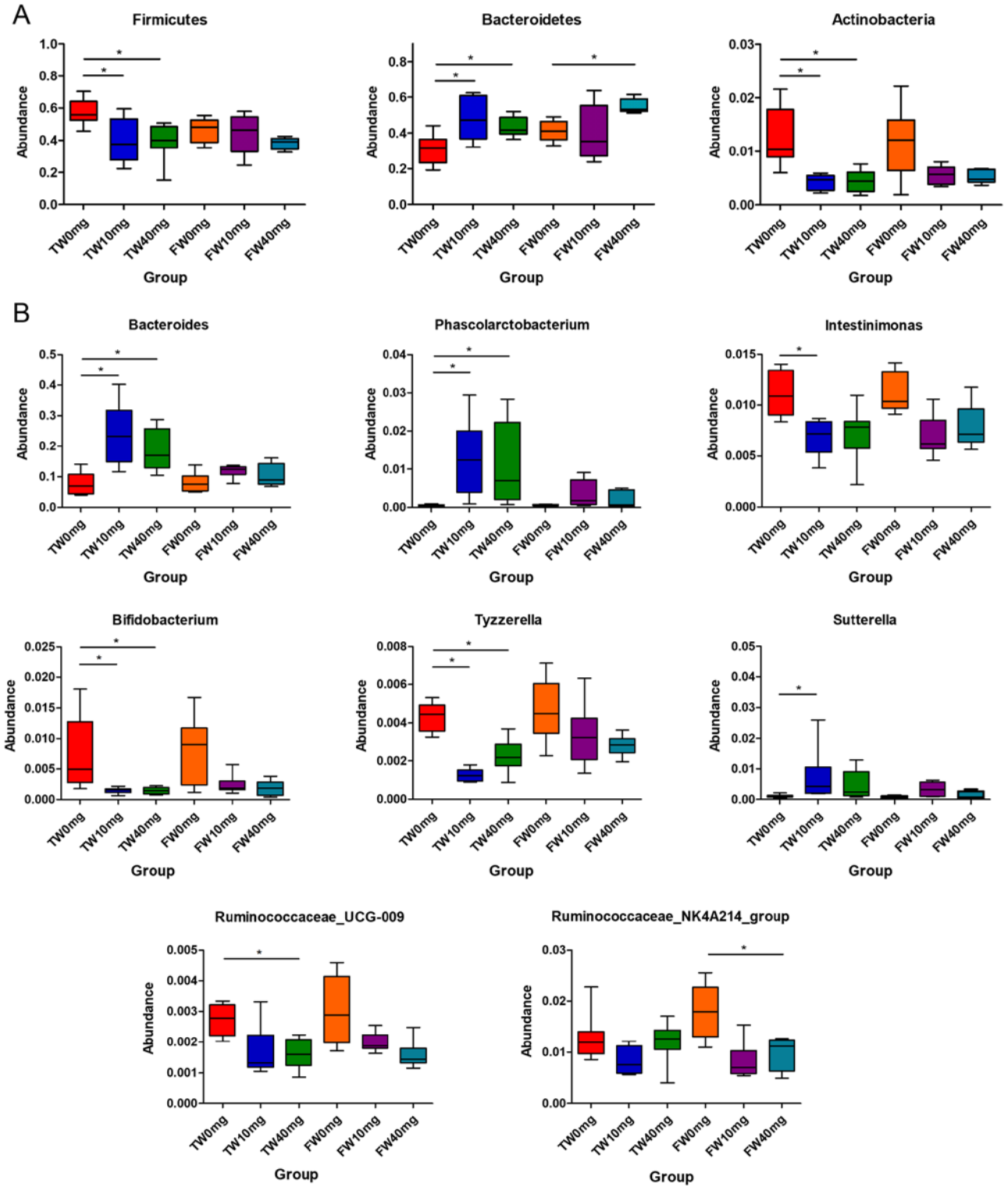

Figure 3. Major taxonomical profiles differences identification. (A) Differences in the major taxonomical profiles of fecal microbiota among different groups at the (A) phylum level and (B) genus level. P-values were corrected for multiple comparisons with Benjamini and Hochberg false-discovery rate correction (q-value). *q-value <0.05 was considered statistically significant. TW, 2-week SV treatment; FW, 4-week SV treatment.

metabolism pathways (as described below) were emphasized. The abundances of pathways associated with 'oxidative phosphorylation' and 'carbon fixation pathways in prokaryotes' (energy metabolism; Fig. 5C), 'pyruvate metabolism' and 'amino sugar and nucleotide sugar metabolism' (carbohydrate metabolism; Fig. 5D), 'amino acid related enzymes' and 'alanine, aspartate and glutamate metabolism' (amino acid metabolism; Fig. 5E), and 'purine metabolism' and 'pyrimidine metabolism' (nucleotide metabolism; Fig. 5F) were significantly changed in the SV treatment groups compared with in the control groups (TW10mg vs. TW0mg; TW40mg vs. TW0mg; FW40mg vs. FW0mg; $P<0.05$). These results highlighted the importance of the gut microbiota in these metabolic pathways.

Table II. Significant bacterial difference in relative abundance of operational taxonomic units at species level.

Species	Mean abundance (SEM)					
	TW0mg	TW10mg	TW40mg	FW0mg	FW10mg	FW40mg
Bacteroides_caccae	0.002989 ($8.69x10^{-4}$)	$0.010917^{b,g}$ ($1.72x10^{-3}$)	0.001600 ($2.95x10^{-4}$)	0.001655 ($2.80x10^{-4}$)	0.009473^{f} ($9.93x10^{-4}$)	0.001340 ($1.63x10^{-4}$)
Parabacteroides_distasonis	0.014800 ($2.98x10^{-3}$)	$0.039378^{b,g}$ ($5.76x10^{-3}$)	0.020773 ($2.87x10^{-3}$)	0.012071 ($2.19x10^{-3}$)	0.018321 ($3.37x10^{-3}$)	0.019155^{d} ($1.87x10^{-3}$)
Escherichia_coli	0.004394 ($1.20x10^{-3}$)	$0.021633^{b,g}$ ($1.04x10^{-2}$)	0.050662^{a} ($3.36x10^{-2}$)	0.004563 ($1.23x10^{-3}$)	0.019439 ($1.15x10^{-2}$)	0.000941^{e} ($2.05x10^{-4}$)
Parasutterella_secunda	0.001016 ($1.92x10^{-4}$)	$0.006215^{b,g}$ ($2.07x10^{-3}$)	0.003664 ($1.51x10^{-3}$)	0.000748 ($1.21x10^{-4}$)	0.002874^{e} ($7.74x10^{-4}$)	0.001141 ($3.88x10^{-4}$)
Bacteroides_intestinalis	0.003002 ($1.99x10^{-4}$)	$0.027231^{c,g}$ ($4.39x10^{-3}$)	$0.012077^{c,g}$ ($3.08x10^{-3}$)	0.004957 ($8.20x10^{-4}$)	0.009997^{d} ($1.75x19^{-3}$)	0.004368 ($9.19x10^{-4}$)
Lachnospiraceae_bacterium_COE1	0.004045 ($6.14x10^{-4}$)	$0.001240^{b,g}$ ($4.21x10^{-4}$)	$0.001582^{b,g}$ ($2.75x10^{-4}$)	0.001710 ($2.38x10^{-4}$)	0.003581 ($1.37x10^{-3}$)	0.001527 ($3.11x10^{-4}$)
Bacteroides_vulgatus	0.005366 ($8.79x10^{-4}$)	$0.030262^{b,g}$ ($8.68x10^{-3}$)	$0.031679^{c,g}$ ($9.06x10^{-3}$)	0.005634 ($5.61x10^{-4}$)	0.005838 ($9.13x10^{-4}$)	0.009851 ($2.95x10^{-3}$)
Bacteroides_fragilis	0.001048 ($2.80x10^{-4}$)	$0.007516^{c,g}$ ($1.12x10^{-3}$)	0.012006^{a} ($7.10x10^{-3}$)	0.00124 ($3.03x10^{-4}$)	0.004647^{e} ($1.03x10^{-3}$)	0.000915 ($2.30x10^{-4}$)
Clostridiales_bacterium_CIEAF_020	0.002924 ($7.63x10^{-4}$)	0.000698^{a} ($3.70x10^{-4}$)	$0.000414^{c,g}$ ($1.11x10^{-4}$)	0.003177 ($1.09x10^{-3}$)	0.001118 ($5.94x10^{-4}$)	$0.000326^{f,g}$ ($8.39x10^{-5}$)
Bacteroides_uniformis	0.004105 ($6.42x10^{-4}$)	$0.020937^{c,g}$ ($6.78x10^{-3}$)	$0.013791^{b,g}$ ($4.62x10^{-3}$)	0.003054 ($2.98x10^{-4}$)	0.006017^{d} ($1.28x10^{-3}$)	0.004761 ($1.16x10^{-3}$)

$^{a}P<0.05$, $^{b}P<0.01$ and $^{c}P<0.001$ vs. TW0mg. $^{d}P<0.05$, $^{e}P<0.01$ and $^{f}P<0.001$ vs. FW0mg. ^{g}q-value <0.05 was considered as statistically significant. Data are presented as the mean ± SEM (n=8). P-values were corrected for multiple comparisons with Benjamini and Hochberg false-discovery rate correction (q-value). TW, 2-week SV treatment; FW, 4-week SV treatment.

Discussion

Statin therapy has different lipid-lowering effects on hyperlipidemia in different individuals, while most efforts to understand statin pharmacodynamics have focused on genetic polymorphisms (41,42). However, growing evidences have suggested that the gut microbiome contributes to the variability in statin metabolism and statin response (19,43). A previous study reported that SV is metabolized by anaerobic bacteria in human fecal suspension (19). A recent study has revealed that antibiotic-modulated gut microbiota could attenuate the hypolipidemic effect of SV in high-fat diet (HFD)-fed mice (44). These results highlighted the potential interaction between gut microbiota and SV, both *in vivo* and *in vitro*. Although SV is considered one of the representative examples of microbial impact on drug bioavailability, the molecular characterization of gut microbiota in response to SV in high-lipid diet-induced hyperlipidemic rats is not fully understood. In the present study, the altered compositional and functional characteristics of gut microbiota in response to SV treatment were observed in high-lipid diet-fed rats.

The community richness of gut microbiota was calculated by the OTUs, Chao1 and ACE indices, while community diversity was explored by the Shannon and Simpson indices. As shown by the decrease in richness and diversity indices, SV therapy reduced not only the total number of species in gut microbiota, but also the heterogeneity in the 2-week treatment groups. In the 4-week treatment groups, no significant change was observed in the richness indices, whereas a significant decrease was observed in diversity indices. A previous 1-week SV study did not report appreciable changes in total bacteria by quantitative PCR (45). A previous 8-week SV study revealed that the hypolipidemic effect of SV is associated with the altered composition of the gut microbiota (44). The influence of SV (2- and 4-week therapy) on gut microbiota lacks information and is not fully understood. In the present study, the V3-V4 region of the 16S rRNA gene was sequenced following 2 and 4 weeks of SV treatment, and changes in richness and diversity of gut microbiota were identified. Consistent with the present study, Liu *et al* (43) and Sun *et al* (46) reported that variation in community richness and diversity was identified between statin-sensitive and -insensitive patients, which may suggest that the complexity of the fecal microbiome is closely associated with SV therapeutic actions. Therefore, the community composition of gut microbiota was analyzed at the phylum, genus and species levels, and the variations among groups were observed.

Recent studies have reported that, at the phylum level, *Bacteroidetes* and *Firmicutes* are predominant in the gut following statin treatment during HFD (44,47). In accordance with previous reports, the present data also demonstrated that *Firmicutes* and *Bacteroidetes* were the dominant phyla in all samples. From the co-occurrence networks, the increased positive correlation within the phylum *Firmicutes* suggested that

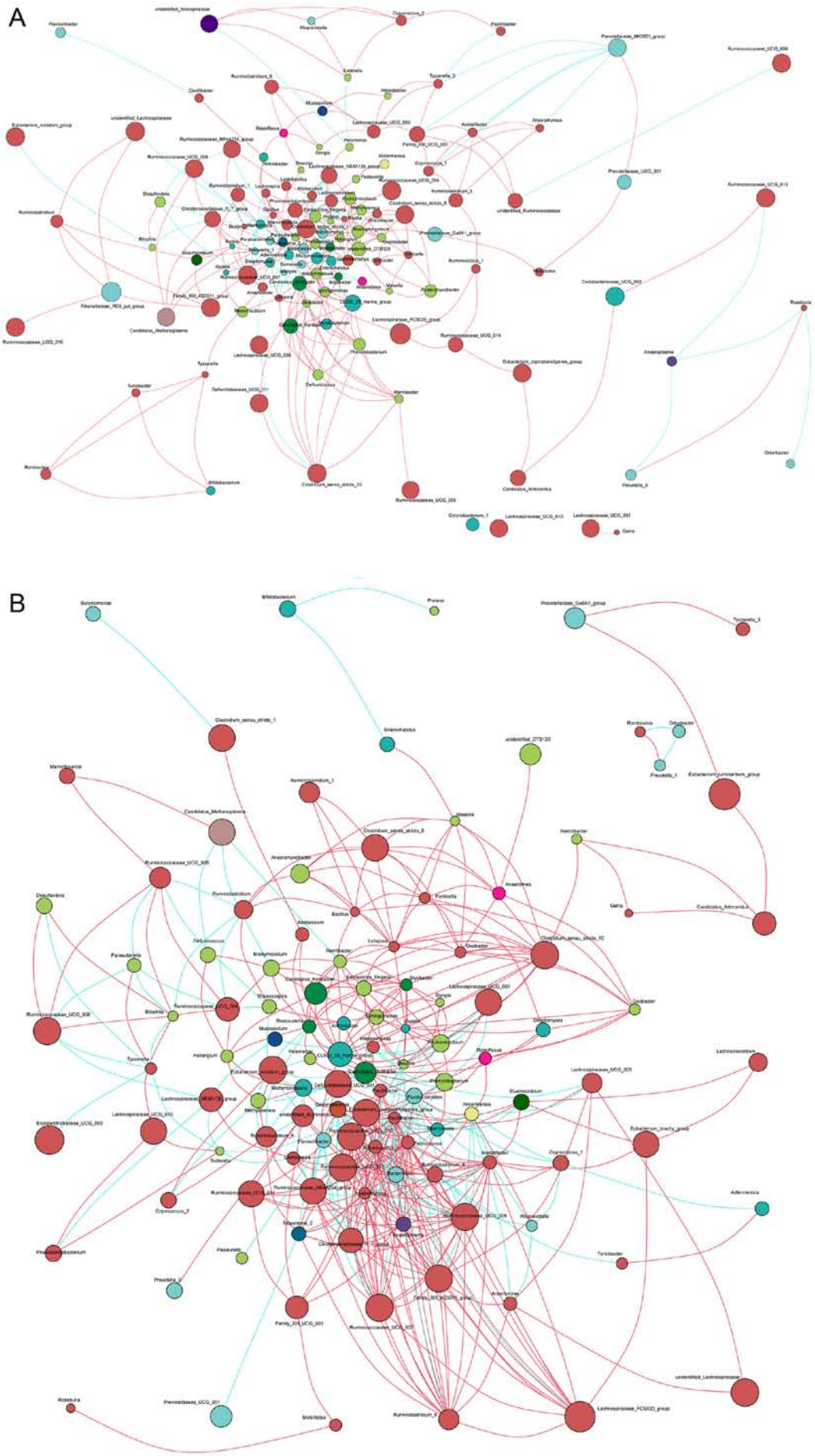

Figure 4. Co-occurrence networks of bacterial genera. Co-occurrence network of bacterial genera in group (A) TW10mg and (B) TW10mg.

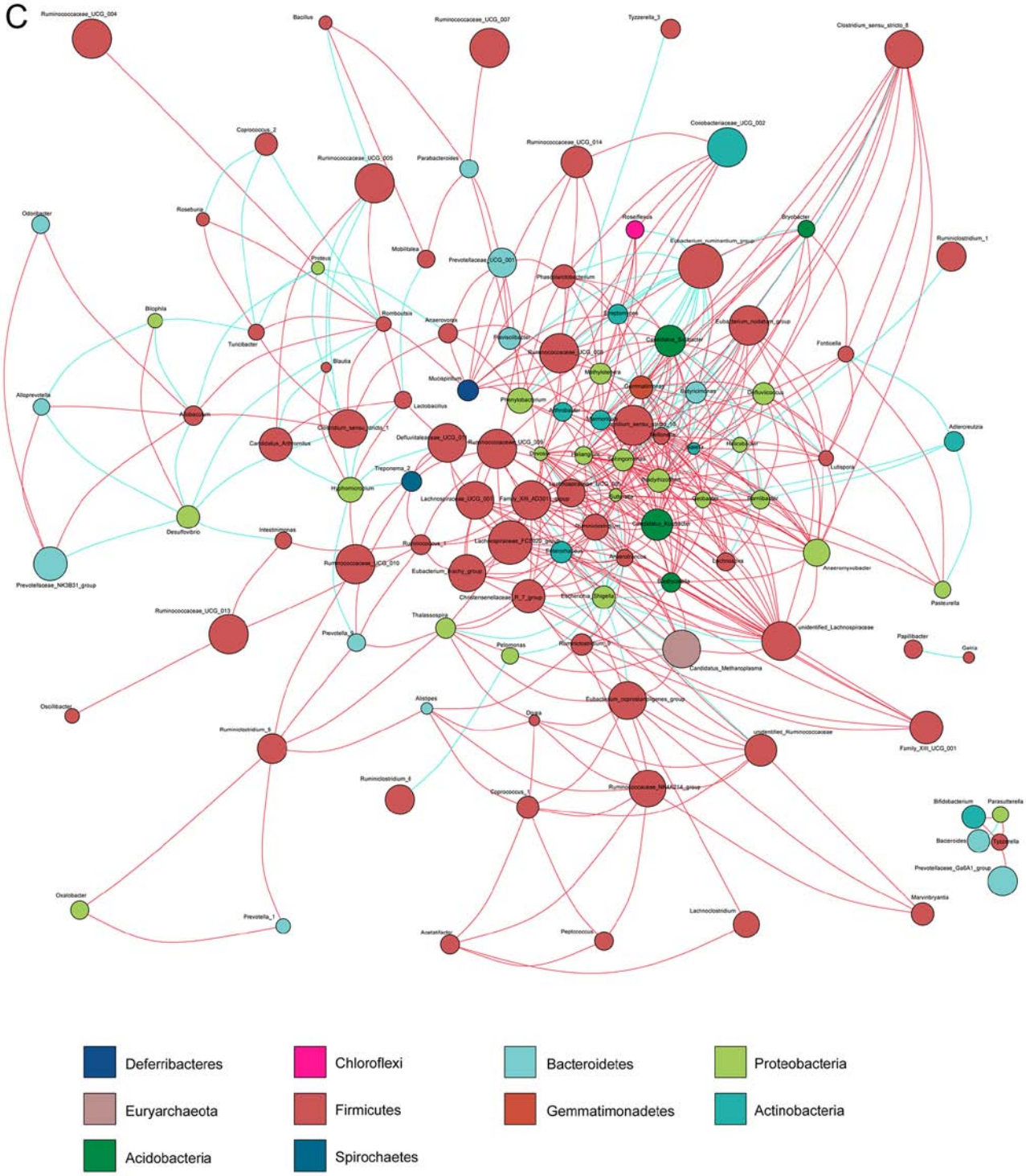

Figure 4. Continued. Co-occurrence networks of bacterial genera. Co-occurrence network of bacterial genera in group (C) TW40mg. Different nodes represent different genera. The nodes sizes are proportional to the average relative abundance of genera. The nodes with the same color are from the same phylum. The color of the line corresponds to the correlation (red is positively correlated; blue is negatively correlated). TW, 2-week SV treatment.

the 2-week SV treatment induced strong interactions between *Ruminococcaceae*, *Family_XIII*, *Lachnospiraceae* and other bacteria within the phylum *Firmicutes*. This result indicated that these associated intestinal floras may by a novel therapeutic target for the improvement of SV efficacy. The abundance of *Bacteroidetes* suggested that a higher dose of SV treatment could cause the enrichment of *Bacteroidetes* in hyperlipidemic rats following 4 weeks of treatment. A decrease in the count of *Firmicutes* and an increase in that of *Bacteroidetes* caused the *Firmicutes* to *Bacteroidetes* ratio (F/B ratio) to drop from 2.05 to 0.70 following SV treatment. Consistent with the findings of the present study, a decreased F/B ratio induced by other two statins (atorvastatin and rosuvastatin) treatment was also observed (47). Since the F/B ratio is associated with metabolic disorders and is broadly considered to be an indicator of gut microbiota dysbiosis (48), the changes in *Firmicutes* and *Bacteroidetes* clearly suggested that SV treatment contributed to the decline of the F/B ratio in a high-lipid model, helping the

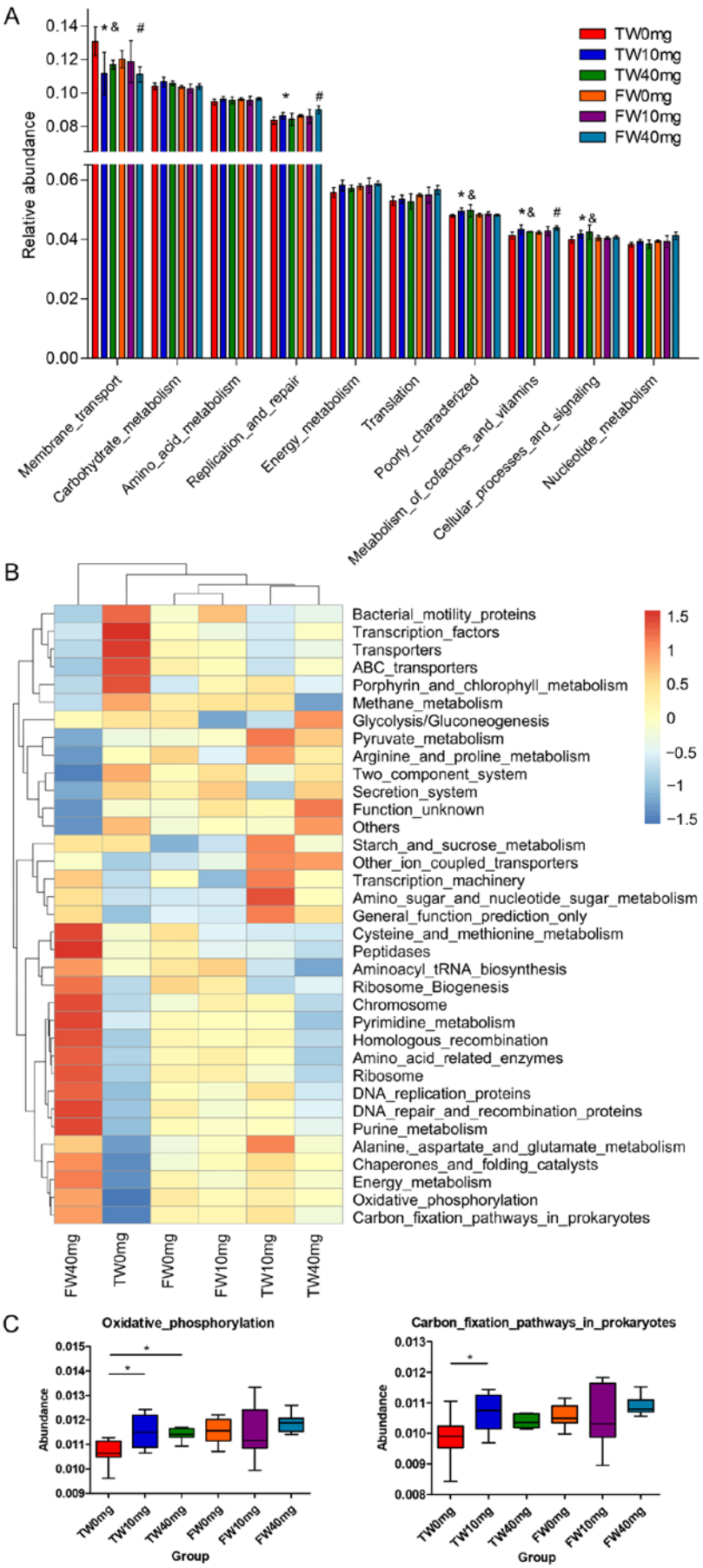

Figure 5. Bacterial gene functions were predicted from 16S rRNA gene-based microbial compositions using the PICRUSt algorithm. (A) KEGG pathway categories in each group at level 2. *P<0.05 (TW10mg vs. TW0mg). &P<0.05 (TW40mg vs. TW0mg). #P<0.05 (FW40mg vs. FW0mg). P<0.05 was considered to indicate a statistically significant difference. (B) KEEG pathway categories in each group at level 3 are shown in a heatmap. KEGG pathways associated with (C) energy metabolism ('Oxidative phosphorylation' and 'Carbon fixation pathways in prokaryotes').

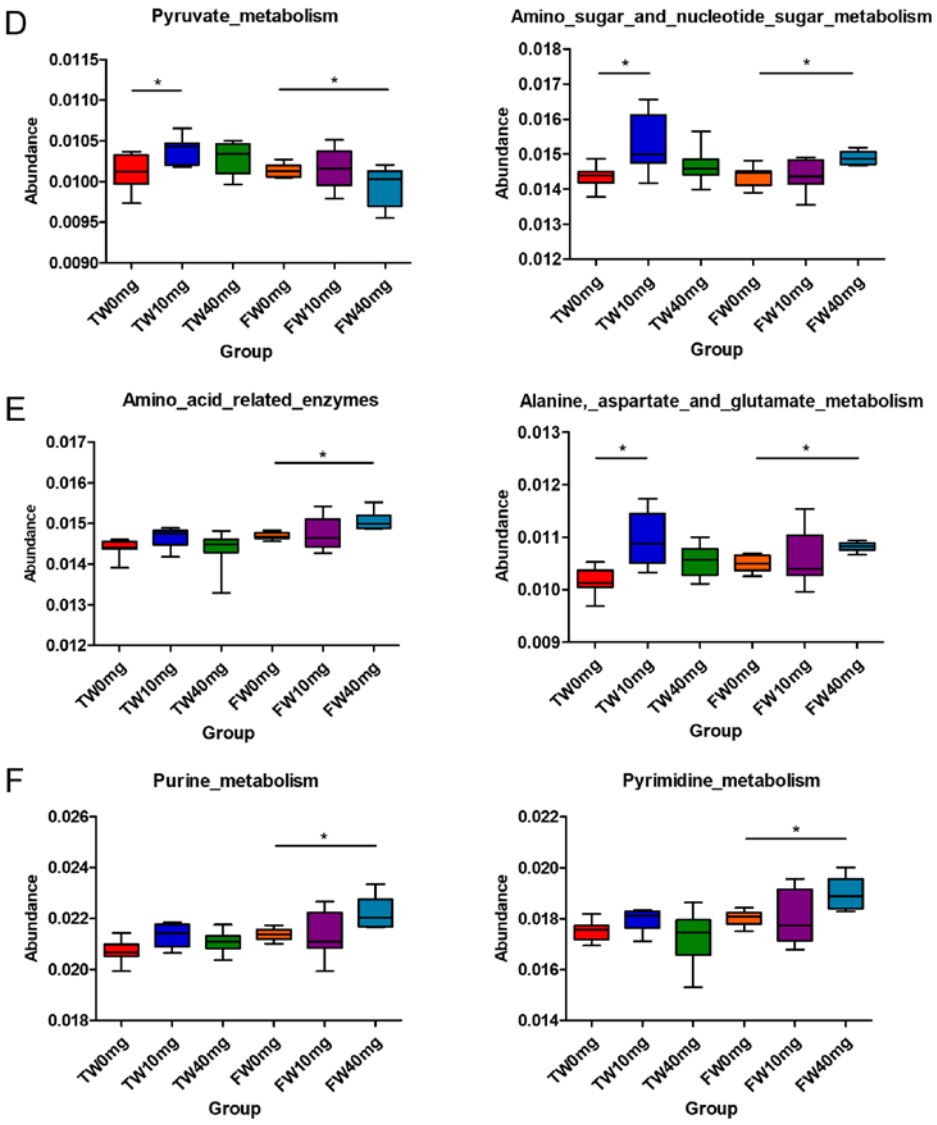

Figure 5. Continued. KEGG pathways associated with (D) carbohydrate metabolism ('Pyruvate metabolism' and 'Amino sugar and nucleotide sugar metabolism'), (E) amino acid metabolism ('Amino acid related enzymes' and 'Alanine, aspartate and glutamate metabolism'), and (F) nucleotide metabolism ('Purine metabolism' and 'Pyrimidine metabolism'). Kruskal-Wallis followed by Dunn's post hoc test was used to test significant differences. *P<0.05. KEGG, Kyoto Encyclopedia of Genes and Genomes; TW, 2-week SV treatment; FW, 4-week SV treatment.

gut microbiota regain its balance. The decrease in *Firmicutes* and the increase in *Bacteroides* in the two control groups (TW0mg and FW0mg) may be due to the high-lipid diet. This was also reported by Wu *et al* (49) and Gentile and Weir (50).

At the genus level, *Ruminococcaceae_NK4A214*, *Ruminococcaceae_UCG-009* and *Intestinimonas* within the family *Ruminococcaceae* were decreased following SV treatment in the present study. It has been reported that the abundance of *Ruminococcaceae* is associated with a positive therapeutic effect of two statins, rosuvastatin (43) and atorvastatin (46). The results of the present study provided more evidence that demonstrated the interaction between *Ruminococcaceae* and statins. As shown in the co-occurrence networks, higher positive correlations of genera *Ruminococcaceae_NK4A21*, *Ruminococcaceae_UCG-004*, *Ruminococcaceae_UCG-009*, etc. were observed in the 2-week treatment groups, suggesting that the effect of SV may improve the interaction among these bacterial floras. *Ruminococcaceae* has been reported to be positively associated with the production of short chain fatty acids (SCFAs) (51) and bile acids (52), and possesses an extraordinary capacity for generating SCFAs and glucose (53). The genus *Tyzzerella* has been identified to be enriched in patients with a high cardiovascular disease risk profile (54); however, in the present study, a decreased level of *Tyzzerella* was observed in rats following SV treatment, suggesting that SV may reduce the risk of cardiovascular disease by influencing the genus *Tyzzerella* during the regulation of dyslipidemia. By contrast, the genus *Phascolarctobacterium* within the phylum *Firmicutes* was markedly increased following SV treatment. This genus is associated with both insulin sensitivity and secretion, and involved in the carbohydrate metabolism in overweight adults (55). This may be due to the production of SCFAs, including acetate and propionate (56). Briefly, the aforementioned *Firmicutes* members, which were identified to be significantly altered in the present study, suggested their potential associations with the metabolites of SCFAs and glucose. Since SCFAs regulate not only the gut barrier function but also lipid, glucose and cholesterol metabolism,

changes in the SCFAs profile could impact the physiology of the host (57). These associations suggest that *Firmicutes* members considerably influence hypolipidemic drug efficacy or drug response by affecting the absorption of nutrients and maintaining energy balance.

At the species level, the present study detected that the proliferation of the genus *Bacteroides* was due to the expansion of the species, including *Bacteroides_caccae*, *Bacteroides_fragilis*, *Bacteroides_vulgatus* and *Bacteroides_uniformis*, as well as *Bacteroides_intestinalis*. As already reported, *Bacteroides uniformis* is associated with hepatic G6pase and farnesoid X receptor (FXR) (58). This association suggests a potential mechanisms through which this bacterium improves glucose tolerance (59). In addition, the species *Bacteroides fragilis* is positively associated with fasting blood glucose (60). *Bacteroides vulgatus* may also reduce gut microbial lipopolysaccharide production and inhibit atherosclerosis (61). Furthermore, Wang *et al* (62) reported that *Parabacteroides distasonis* within the phylum *Bacteroidetes*, as a promising probiotic, could modulate the host metabolism to alleviate obesity and metabolic dysfunctions by generating the secondary bile acids and succinate. Consequently, it has been accepted that these aforementioned microbial taxa at the species level may affect physiological functions in the host and serve a role in the statins-induced metabolic improvements.

The likely functional capacity of microbiota genes that are modulated by SV treatment were predicted using PICRUSt. The PICRUSt approach may cause potential biases due to the infrequent update of GreenGene, and some OTUs could not be matched against GreenGene (63). Additionally, functional profiles of bacterial communities were based on the 'common ancestor gene'. PICRUSt was based on the OTU tree to deduce the function of the common ancestor gene. There is an association between the phylogenetic relatedness of organisms and their complement of functional genes (39). Although potential biases exist, PICRUSt has been applied to samples collected from a wide range of habitats, including the human gastrointestinal tract (64). It must be remembered that PICRUSt predicts the functional attributes of a microbiome rather than identifying them directly from DNA sequences, the predictions made by PICRUSt can serve as useful hypothesis-generating tools and provide alternative ways to probe the structure of the microbiome (39,65). A previous study has demonstrated that the immune system balances the microbiota by directing the metabolism towards oxidative phosphorylation and fatty acids oxidation (66). The SV-associated microbiota likely exhibited enrichment in genes for energy metabolism, including oxidative phosphorylation in the present study. These observations suggested that SV may target the microbes for remediation of the immune system. Additionally, the results demonstrated that the microbiota exhibited enrichment in genes for the alanine, aspartate and glutamate metabolism pathway following SV treatment. Consistent with the present study, previous studies have reported that acetic acid could be produced by gut bacteria like *Bacteroides spp.*, *Bifidobacterium spp.* and *Ruminococuus spp.* (67,68). In addition, propionic acid could be produced from alanine and aspartate, and acetic and butyric acid could be produced from glutamate (69). It is known that acetic, butyric and propionic acid are the main ingredients of SCFAs (70). SCFAs inhibit insulin signal transduction and control energy consumption by activating free fatty acid receptor 3 and free fatty acid receptor 2 (71). Of note, intestinal bacteria like *Ruminococcaceae*, *Phascolarctobacterium* and *Bacteroides*, which are important sources of SCFAs (72), showed significant changes following SV treatment in the present study. Previous studies have demonstrated that gut microbiota regulates triglyceride, cholesterol and bile acid homeostasis metabolism by influencing FXR (73,74). In the present study, the genera *Bacteroides* and *Bifidobacterium*, which have been identified to be associated with bile salt hydrolases (BSH) activity (75), were significantly altered. The BSH-catalyzed step is considered the 'gateway reaction' of microbiota-mediated bile acid metabolism (76). These findings suggested that the effect of SV on metabolic improvements could be explained by altered gut microbiota.

In conclusion, the findings of the present study revealed that SV treatment could reduce gut microbial community richness and diversity. The SV treatment also contributed to the remodeling of the fecal bacterial community composition, mainly including a decrease in the phylum *Firmicutes* and an increase in the phylum *Bacteroidetes*. Furthermore, the SV-associated modulation in gut microbiota genes has a profound influence on the energy, carbohydrate, amino acid and nucleotide metabolism pathways. Understanding the causal links between gut microbiota gene content and associated-metabolic activities may provide novel insights into potential drug targets for preventing hyperlipidemia.

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Availability of data and materials

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

Authors' contributions

SZ designed and executed the experiments, analyzed the data and wrote the manuscript. HL, LY, JZ, LH, RL, RW, YS, NM and SU helped perform the experiments. JX conceived and designed the study, critically reviewed and drafted the manuscript. All authors take responsibility for the integrity of the work as a whole. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and performed with the approval of the Ethical Committee of Xi'an Jiaotong University School of Medical Sciences (approval no. 2017-288).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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