Supplementation of triple viable probiotics combined with dietary intervention is associated with gut microbial improvement in humans on a high-fat diet

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Abstract. Numerous animal studies have demonstrated that oral probiotics may have a beneficial role in preventing obesity, inflammatory bowel disease and even colorectal cancer, which are all associated with a high-fat diet (HFD). However, the underlying beneficial effects of combined probiotic and dietary intervention on the gut microbiota of 'non-patient' individuals previously on an HFD have yet to be fully elucidated. In the present study, fecal samples were obtained from 36 volunteers on a high-fat diet and after dietary intervention for 4 months, and 16S rDNA sequencing was applied to identify how probiotics and dietary intervention had altered the composition of the microbiota. The results demonstrated that probiotics treatment and dietary intervention in combination raised the diversity of lumen microbes compared with their individual applications. A markedly separated distribution (β-diversity) was observed, confirming the difference in gut microbiota composition among the treatment groups. Bacterial taxonomic analysis demonstrated that the relative abundance of 30 species was altered among the groups following dietary intervention and/or probiotic supplementation. The majority of the species that exhibited a population increase belonged to two butyrate-producing families, *Ruminococcaceae* and *Lachnospiraceae*, whereas the species with reduced populations mainly belonged to the *Bacteroidaceae* family. Collectively, these results suggest that combined probiotic and dietary intervention is able to improve the gut microbiota composition of human subjects on an HFD.

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Introduction

Approximately 10^3 distinct bacterial species, the total number of which is >10-fold that of eukaryotic cells, colonize in the human gastrointestinal tract (1,2). The commensal intestinal microbiota fulfills important roles in digestion and absorption of nutrients, the composition of the gut mucosal barrier, host metabolism and the innate immune system (3-7). A healthy gut microbiota maintains homeostasis within the host, which may be crucial to the normal operation of other vital organs, including the liver (8-11) and the brain (12-15). Accumulating evidence suggests that long-term dietary habits may result in various gut microbiota, comprising different bacterial ecosystems, which are known as enterotypes (16-18). A Western-style diet, high in total and saturated fat, is relevant to the prevalence of numerous illnesses and conditions, including obesity, type 2 diabetes, inflammatory bowel disease (IBD) and digestive tract cancers, probably due to an imbalance in gut microbial populations. A study by Kim *et al* (19), which revealed that a high-fat diet (HFD) induced low-grade inflammation, demonstrated the pro-inflammatory effects of energy-dense diets on vagal gutbrain communication. David *et al* (20) reported that increases in the growth of the sulfite-reducing pathobiont *Bilophila wadsworthia* in the presence of a diet rich in animal fats supported an association between dietary fat, bile acids and the outgrowth of microbes, which was able to trigger IBD. Schulz *et al* (21) demonstrated that an HFD accelerated tumor progression in the small intestine of K-ras^{G12Dint} mice, which was independent of the occurrence of obesity. Ou *et al* (22) reported that the effects of dietary fat on the occurrence of colorectal cancer (CRC) may be indirectly mediated by secondary bile acids, which are produced via the enterobacterial 7α-dehydroxylation of primary bile acids and are considered to be potential carcinogens in the etiology of CRC. However, a direct association between diet, the intestinal microbiota and the pathogenesis of CRC has yet to be conclusively demonstrated, even in animal models. Of note, a number of epidemiological studies have demonstrated that excessive intake of animal fat is associated with an increased risk of colon cancer, which may promote the expansion of sulfate-reducing bacteria that are thereby able to produce genotoxic agents, including hydrogen sulfide.

Probiotics consist of live beneficial bacterial species for promoting health of the host, typically lactobacilli and bifidobacteria, and these directly improve the composition of the colonic microbiota (23). A study by Hu *et al* (24) that employed a high cholesterol diet-fed rat model suggested that two *Lactobacillus* strains exerted a positive effect on lipid metabolism. *Lactobacillus delbrueckii* and *Bifidobacterium animalis* var. *lactis* administered alone were indicated to ameliorate colonic preneoplastic lesions in mice, although their administration in combination did not prove to be effective (25). Bertkova *et al* (26) demonstrated that probiotic *Lactobacillus plantarum* together with bioactive compounds was able to suppress colon carcinogenesis in N,N-dimethylhydrazine-induced rats. In China, capsules containing live, combined *Lactobacillus*, *Bifidobacterium* and *Enterococcus* have been widely used as probiotics in clinical settings, including the treatment of non-alcoholic fatty liver disease (27), irritable bowel syndrome (28) and even gastrointestinal tumors (29,30), and these probably work via reducing the inflammatory response and forming a biological protective barrier.

Various functional effects of different probiotic strains on the gut microbiota and relevant diseases have been clearly demonstrated. However, certain studies have identified that gut microbiota undergo a less pronounced response to oral probiotics, partly due to inter-individual variation in microbiota composition elicited by dietary habits, in addition to the genetic background, age, other environmental factors, or the application of different analytical methods. In this regard, the concept of three enterotypes was raised by Arumugam *et al* (16), namely *Bacteroides*, *Prevotella* and *Ruminococcus*. In reality, it was difficult to categorize human beings as a particular 'enterotype', since dietary factors may have an impact on gut microbial populations (31,32). A previous study by our group demonstrated a different composition of stool bacterial genera when comparing between individuals on either a high- or a low-fat diet (33). Since diet may shape the composition and function of the gut microbiota, in turn influencing host health, one promising therapeutic strategy aimed at improving health would be to alter the gut microbiome using dietary intervention.

Although the positive effects of probiotics and dietary intervention on IBD and CRC have been broadly demonstrated, comparative studies to identify the efficacy of probiotic supplementation and dietary intervention as prophylactic tools previously under HFD conditions require further investigation ahead of the occurrence of gastrointestinal diseases. Therefore, the present study aimed to assess whether probiotic treatment and dietary intervention exert any impact on the gut microbiota in human subjects on an HFD.

Subjects and methods

Subjects, diets and experimental design. A total of 36 healthy volunteers (age, 45-65 years; males/females, 16/20) from Zhouzhuang Town (Jiangyin City, China), who were consuming an HFD with dietary fat accounted for >40% of total energy (34), were enrolled mainly according to the results of questionnaire and physical examination during the community health survey. All experiments of the present study were approved by the Ethics Committee of Shanghai Tenth People's Hospital (Shanghai, China) and Jiangyin People's Hospital (Jiangyin, China). Written informed consent was obtained from all of the participants prior to their enrolment. Subsequently, these volunteers were randomly assigned to four groups: i) The HFD group, where the HFD was maintained due to their constant habits to establish a control group (n=9); ii) the dietary intervention (DI) group, where the HFD was replaced by a low-fat diet (LFD) in which dietary fat accounted for <40% of total energy (n=9); iii) an HFD + Probiotic group, where the HFD was supplemented with a daily dose of 2 g live combined *Lactobacillus acidophilus* [$\geq 1.0 \times 10^7$ colony-forming units (Cfu)/g], *Bifidobacterium longum* ($\geq 1.0 \times 10^7$ Cfu/g) and *Enterococcus faecalis* ($\geq 1.0 \times 10^7$ Cfu/g) powder (Shanghai Xinyi Pharmaceutical Co., Ltd.) administered orally in water according to the manufacturer's recommendation (n=9); and iv) the DI + Probiotic group, which received a combination of the LFD and the above-mentioned probiotic microorganisms (n=9). No significant differences in daily intake of dietary fiber, or the percentages of calories derived from fat, were identified among the four groups prior to the experiment (Fig. 1). The volunteers also had a similar gender ratio, were of a similar age and body mass index, yielded similar valid sequencing reads for fecal microbiota and had similar gut microbiota compositions at the start of the experiment. These details were presented in Supplementary Table SI. Probiotic treatment and dietary intervention were continued for 4 months (from Jan 1st, 2015 to Apr 30th, 2015), which hopefully allowed sufficient duration for intestinal flora change. All of the volunteers completed the study under strict quality control with dietary changes and medication compliance monitored throughout the study.

Stool collection. A freshly voided stool sample was collected from each subject at the end of the experiment (Apr 30th, 2015), and was snap-frozen in liquid nitrogen. All samples were finally transferred to a deep freezer at -80˚C until the microbiota was analyzed.

Microbiota analysis

DNA extraction. Metagenomic DNA was extracted from each fecal sample using a MicroElute Genomic DNA kit (cat. no. D3096-01; Omega; BioTek, Inc.) according to the manufacturer's protocol. The MicroElute Genomic DNA kit extraction controls were included through the DNA extraction and PCR steps as a negative control. Total DNA was eluted in 50 μl elution buffer using a method modified from the manufacturer's protocol (Qiagen; GE Healthcare) and stored at -20˚C prior to performing qPCR.

PCR amplification and 16S rDNA sequencing. The primers 319F and 806R were used to amplify the double hypervariable V3 and V4 regions of the bacterial 16S rRNA. PCR procedures were as follows: Initial denaturation (30 sec at 98˚C), followed by 35 cycles of amplification including denaturation (10 sec at 98˚C), annealing (30 sec at 54˚C) and extension (45 sec at 72˚C), and then final extension (10 min at 72˚C). The PCR products for each example were normalized using an AxyPrep® TM Mag PCR Normalizer (Axygen Biosciences), followed by purification with AMPure XT beads (Beckman Coulter Genomics),

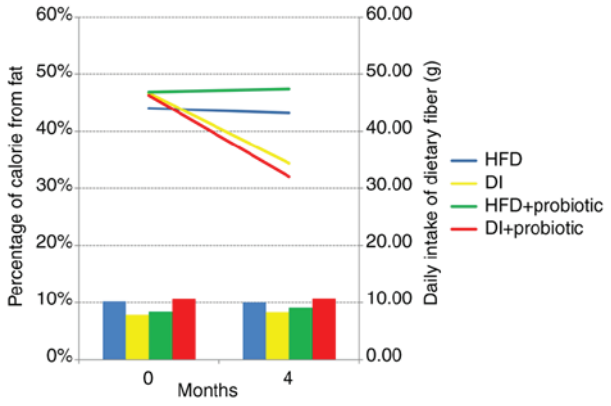

Figure 1. Composition of experimental diet. Dietary fat of the HFD accounted for >40% of total energy, whereas that of the DI was <40%. Furthermore, no significant changes in the daily intake of dietary fiber in any of the groups occurred between day 0 and the end of the experiment (at month 4). HFD, high-fat diet; DI, dietary intervention with low-fat diet.

and quantification using the Illumina Library Quantification kit (Kapa Biosciences). Pyrosequencing was performed using a MiSeq System (Illumina, Inc.).

Bioinformatics analysis. After filtering the raw data, the high-quality sequences were identified, which were subsequently clustered into operational taxonomic units (OTUs) using the CD-hit-est-based clustering method (35). The numbers of the sequences and OTUs for each sample were calculated by using the software PyNAST (http:/qiime.org/pynast/). Subsequently, analysis of the α-diversity (i.e., the mean species diversity in sites or habitats at a local scale), including the Observed species, Shannon, Simpson and Chao1 indexes, was performed. A Venn diagram of the common and unique OTUs among the four groups was constructed using online software (http://bioinfogp.cnb.csic.es/tools/venny/). Unweighted Unifrac distance metrics analysis and principal coordinate analysis (PCoA) were also performed in terms of the matrix of distance to assess the β-diversity (i.e., the ratio between regional and local species diversity). The relative abundance (%) of bacteria at the phylum and species taxa levels in each sample were calculated by using RDP-derived taxonomic communities. A heatmap on species information was constructed using Heml software (version 1.0.3.3) (36).

Statistical analysis. Student's t-test and Kruskal-Wallis one-way analysis of variance with Bonferroni's correction control were performed using SPSS version 19.0 (IBM Corp.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of dietary intervention and oral probiotics on the gut microbial diversity indices in an HFD population. A total of 2,203,533 pyrosequencing reads for the fecal microbiota were analyzed with a mean of 63,573, 57,697, 63,598 and 59,968 reads for the HFD, DI, HFD + Probiotic and DI + Probiotic group, respectively. According to the indices of Observed species, Shannon and Chao1 for stool (Fig. S1), but not according to the Simpson index, the αdiversity of the fecal microbiota of the DI + Probiotic group was higher compared with that of the DI group or the HFD group ($P<0.05$). However, there was no significant difference between the HFD and the DI group, or between the HFD + Probiotic and the DI + Probiotic group. β-diversity analysis revealed that the fecal samples were distributed diffusely and apparent non-overlapping clusters among groups were also formed (Fig. 2).

Effects of dietary intervention and oral probiotics on the gut microbiota composition of an HFD population. The three most abundant phyla in the fecal microbionta, including *Firmicutes*, *Bacteroidetes* and *Proteobacteria*, were not significantly different among the groups at 4 months (Fig. 3A). The relative abundance of the other phyla (mainly including *Actinobacteria* and *Fusobacteria*) was low in all four groups. There was no apparent inter-individual variability at the phylum level within each group (Fig. 3B).

Subsequently, the differences in shared species among groups were analyzed. Overall, there were 467 species detected in stool samples (323, 385, 411 and 418 species in the HFD, DI, HFD + Probiotic and DI + Probiotic group, respectively). When comparing 100% (9/9), 89% (8/9), 78% (7/9), 67% (6/9), 56% (5/9), 44% (4/9) and 11% (1/9) of the populations in each group with each other, the number of species shared among all groups was 5, 9, 22, 43, 74, 109 and 270, respectively. These results suggested that the number of bacterial species shared between the groups is dependent on how many subjects in each group were used for the comparison between the groups (Fig. S2).

Subsequently, the differences in species composition of the fecal microbiota were analyzed. In total, 30 species were different among the four groups, and the majority of these were unclassified with the exception of two distinct species, *Prevotella copri* and *Bacteroides ovatus*. It was revealed that 27 species were altered in subjects receiving dietary intervention and probiotic treatment compared with the other three groups. The relative abundance of 5, 9 and 25 species in the DI, HFD + Probiotic and DI + Probiotic groups, respectively, was significantly different compared with the HFD group ($P<0.05$; Fig. 4). The relative abundance of 17 species, predominantly belonging to the *Ruminococcaceae* and *Lachnospiraceae* families of the order *Clostridiales* and the phylum *Firmicutes*, was increased in subjects from the DI + Probiotic group compared with the HFD group, whereas 8 species were decreased in the DI + Probiotic group, particularly two *Bacteroides* species with relatively higher abundance (*B. ovatus* and one unclassified) belonging to the *Bacteroidaceae* family of the order *Bacteroidales* and the phylum *Bacteroidetes*. Other reduced unclassified species associated with probiotic supplementation and dietary intervention belonged to various different bacterial families: *Flavonifractor*, *Clostridium XVIII*, *Veillonella*, *Anaerostipes* and *Fusobacterium*.

Discussion

In the present study, healthy human subjects who were consuming an HFD were selected as research subjects rather than rodents, e.g. mice or rats, whose confounding factors, including genetic background, age, sex and diet, may be well controlled, and also in preference to human subjects who had

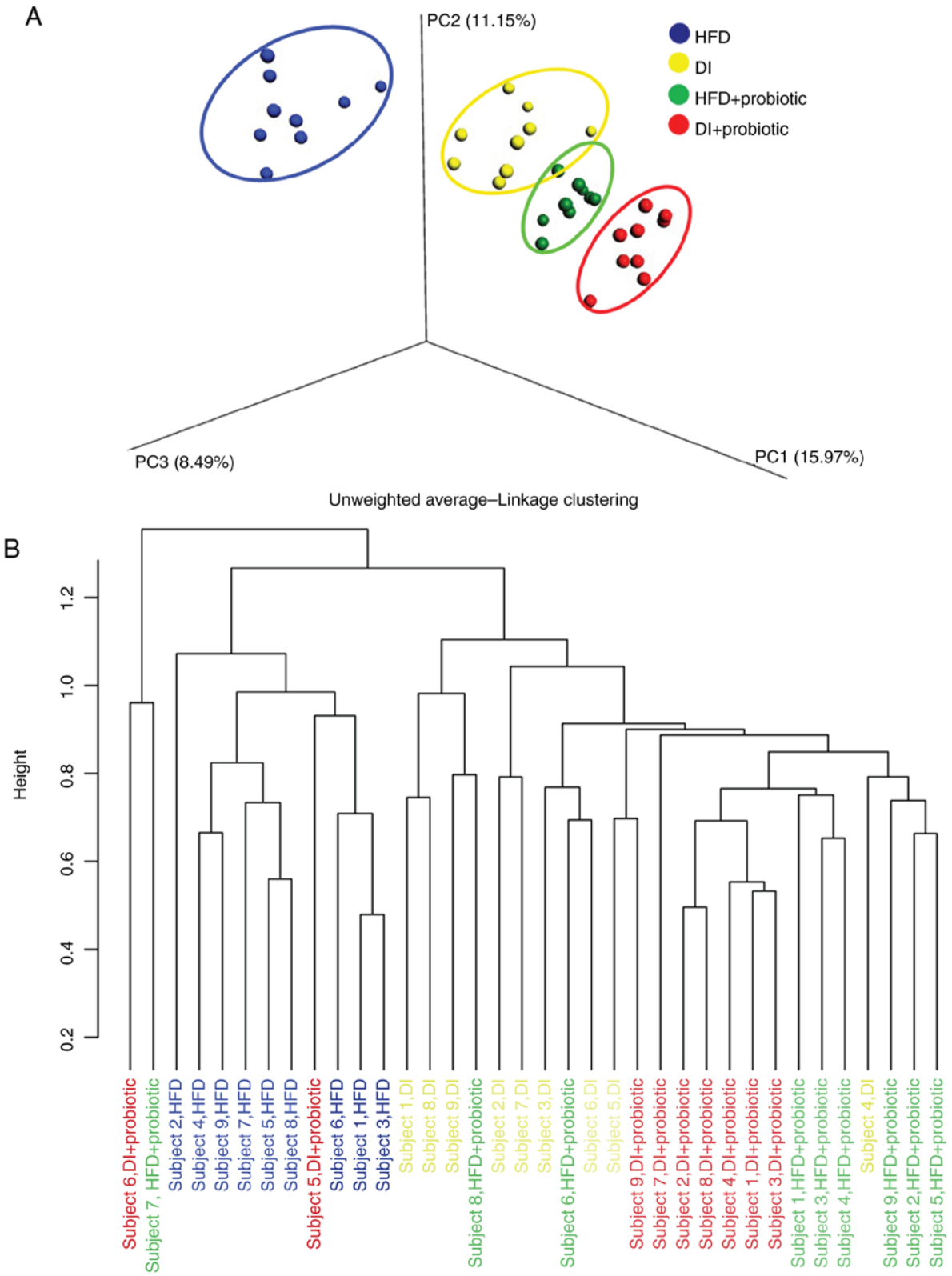

Figure 2. Clustering of samples based on fecal microbiota communities. (A) Principal coordinates analysis of samples from the HFD, DI, HFD + Probiotic and DI + Probiotic groups. (B) Unweighted pairgroup method with arithmetic mean of samples from the HFD, DI, HFD + Probiotic and DI + probiotic groups. Probiotics included *Bifidobacterium longum*, *Lactobacillus acidophilus* and *Enterococcus faecalis*. PC1, PC2 and PC3, three eigenvalues calculated by distance matrix of fecal samples, represent the top three principal coordinate components explaining as much of the variability in the data as possible. HFD, high-fat diet; DI, dietary intervention with low-fat diet.

already incurred disease entities, including obesity, type 2 diabetes and intestinal disorders. Furthermore, under clinical conditions, not all individuals on an HFD develop obesity, hyperlipemia, IBD and intestinal tumors, as other factors are involved, including genetic background, dietary pattern, energy expenditure, metabolic capability and intestinal barrier

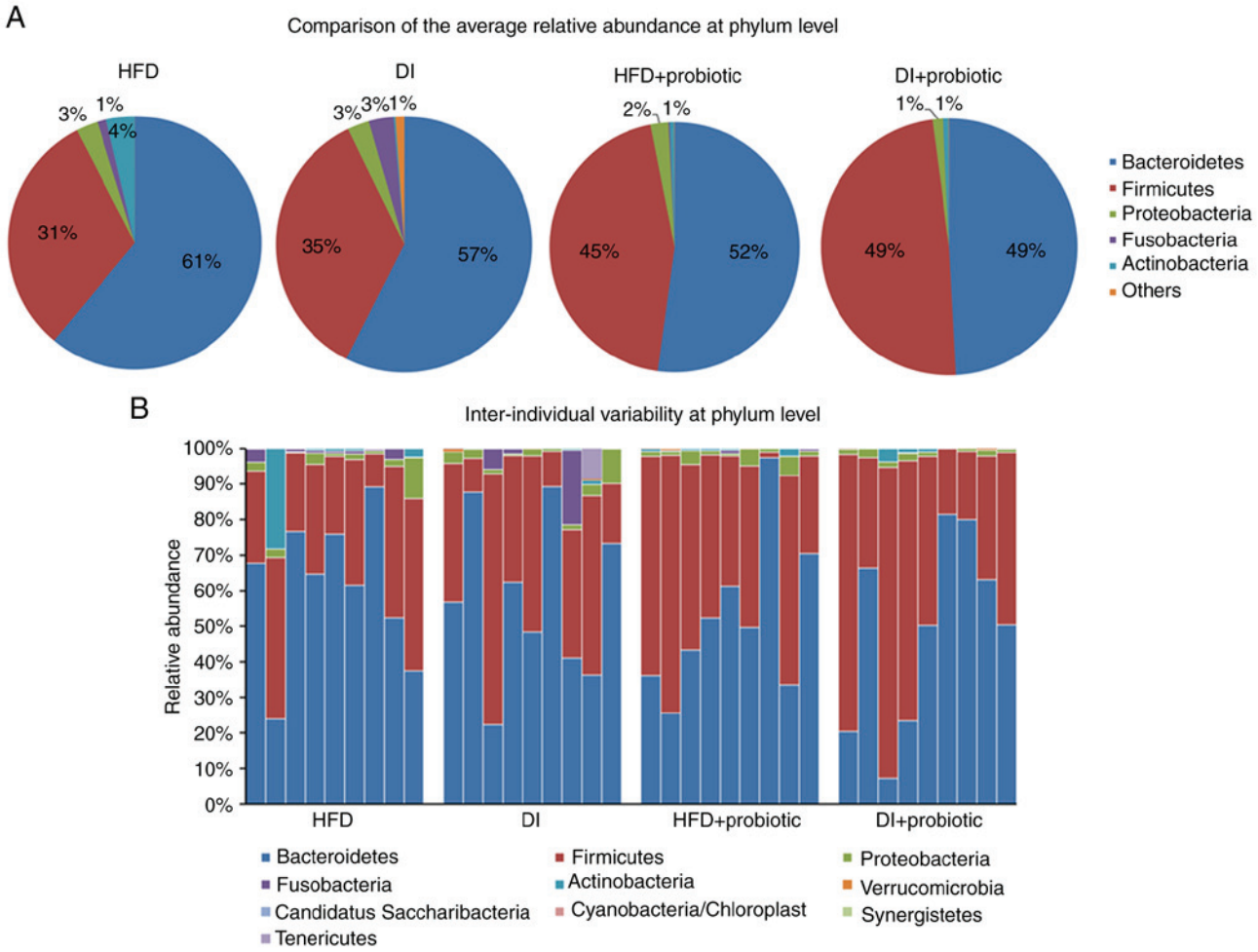

Figure 3. Effect of dietary intervention and probiotic supplementation on the fecal microbiota composition at the phylum level. (A) Pie charts displaying the average relative abundance of stool phyla. (B) Bar graphs indicating the inter-individual variability of stool bacteria at the phylum level. HFD, high-fat diet; DI, dietary intervention with low-fat diet.

state. Although a large number of reviews have described how probiotics are regarded as a gut microbiota-targeted therapy to treat HFD-induced obesity, type-2 diabetes and gastrointestinal diseases (23,37-39), the role of combined oral probiotics and dietary intervention in the modulation of the gut microbiota of healthy populations consuming an HFD remained to be fully elucidated. The results of the present study indicated that probiotics may provide a means of improving the gut microbiota of HFD populations naturally, and that this effect may be enhanced by combining probiotics with dietary intervention. To a certain extent, the gut microbiota may be a suitable target of therapeutic intervention to prevent those individuals on an HFD from developing the above-mentioned diseases.

First, the present results indicated that the diversity of gut microbiota of individuals receiving an HFD may be altered after probiotic supplementation with three live bacterial strains and dietary intervention. Probiotic supplementation in two groups led to a more distinct microbial clustering that was closer in the PCoA plot, irrespective of dietary patterns. This combined effect on adults on an HFD has been rarely reported. However, the topic of changes in the diversity of the gut microbiota remains controversial (40). It was found that daily consumption of yogurt including lactobacilli and bifidobacteria by healthy medical students increased the α-diversity of the intestinal microbiome (41). However, other studies were unable to identify any significant changes in gut microbial diversity, either on the basis of terminal restriction fragments or next-generation sequencing analysis, when comparing day 0 and day 42 of yogurt consumption (42). Therefore, the overall composition of the gut microbial community and its diversity may be governed and affected by the employed detection method of the bacteria. On the other hand, a systematic review of randomized controlled trials were observed no effects of probiotics on the fecal microbiota composition in terms of α-diversity in any of the included studies when compared with a placebo, whereas only one study identified that probiotics were able to significantly modify the fecal bacterial community in terms of β-diversity (43). The consumption of *Lactobacillus casei* Zhang *et al* (44) markedly altered the composition of the intestinal microbiota and the gut microbiota diversity. Park *et al* (45), also demonstrated that mice in a probiotic treatment group had a lower gut microbiota diversity. Although low gut microbiota diversity is usually a hallmark of intestinal dysbiosis, factors including ecological stability, idealized composition or favorable functional profile have recently been suggested as hallmarks of a healthy gut microbiota (46). Certain probiotic strains may diminish the diversity of the gut microbiota either by production of

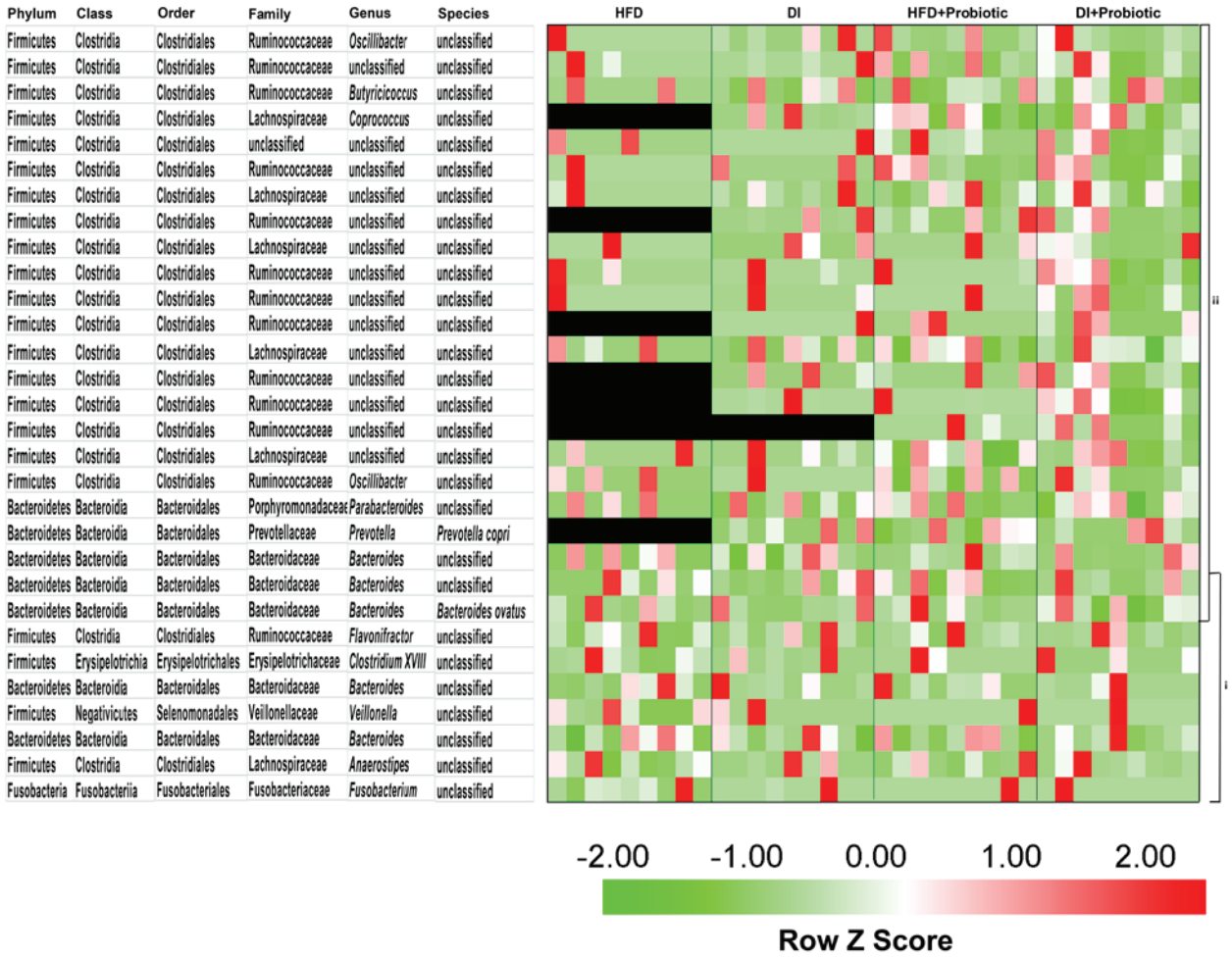

Figure 4. Effect of dietary intervention and probiotic supplementation on the fecal microbiota composition at the species level. The relative abundance of altered species was visualized using a heatmap. Data are represented as row-scaled Z-scores. The black bars represent absent species. i and ii indicate species reduced and elevated in the DI, HFD + Probiotic and DI +Probiotic groups when respectively compared with the HFD group. Probiotics included *Bifidobacterium longum*, *Lactobacillus acidophilus* and *Enterococcus faecalis*. HFD, high-fat diet; DI, dietary intervention with low-fat diet.

anti-microbial peptides, or alternatively, by increasing competition for nutrients, processes that are able to reduce microbial growth. Hanifi *et al* (47) demonstrated that supplementation with *Bacillus subtilis* R0179 did not appear to reverse the overall microbiota diversity, as it simultaneously inhibited the growth of certain opportunistic pathogens. In the present study, it was observed that simply replacing an HFD with an LFD did not alter the α-diversity of the fecal microbiota. A murine (RELMβ) knockout model study revealed that alterations in gut microbiome composition induced by dietary fat were independent of obesity (48). In the present study, it was revealed that ~50% of all detected microbial species were shared by 44% of the volunteers, regardless of diet or treatment. However, divergent results have been identified in other studies. Intestinal microbiota diversity was reduced in HFD- and high sugar diet-fed mice, whereas a control diet was able to prevent these changes. Heinsen *et al* (49) demonstrated that a very low-calorie diet beneficially altered the gut microbiome diversity in obese human subjects, but that these changes were not sustained during weight maintenance.

At the phylum level, the ratio of *Bacteroidetes* to *Firmicutes* was indicated to be decreased in diet-induced obesity, which could be reversibly increased by diet adaptation (50,51). By contrast, various studies have suggested that the ratio of *Bacteroidetes to Firmicutes* is not a contributing factor in human obesity, and it appears not to be associated with diet (52,53). In line with this, in the present study, no significant differences were observed in the relative abundance of *Bacteroidetes* and *Firmicutes* among the four treatment groups, which may be due to differences in genetic background, age and sex. Furthermore, dietary intervention and probiotics supplementation were unable to change the bacterial composition at the phylum level, since *Bacteroidetes* and *Firmicutes* are the phyla predominantly present in the gut microbiota of humans (53-56).

At the species level, significant differences among the treatment groups were identified in the present study, which was consistent with the results of the animal study by Park *et al* (45). The relative abundance of 30 species was altered by dietary intervention and/or probiotic supplementation, 25 of which were changed in subjects who received dietary intervention and probiotic treatment. In addition, increased unclassified species mainly belonged to two butyrate-producing families, *Ruminococcaceae* and *Lachnospiraceae* (57), for which three taxa have been identified at the genus level, including *Oscillibacter*, *Butyricicoccus* and *Coprococcus*, which

may be beneficial for colonic health (58-63). One study has indicated that two *Lactobacillus* strains, *Lactobacillus plantarum* HAC01 and *L. rhamnosus* GG, exert a beneficial effect on *Lachnospiraceae* and *Ruminococcaceae* at the bacterial family level, rather than *Firmicutes* and *Bacteroidetes* at the phylum level (64). The relative abundance of the *Lachnospiraceae* (phylum *Firmicutes*) was significantly higher in the *Lactobacillus*-treated groups compared with that in the PBS-treated control group. Amelioration of obesity-associated dysbiosis by alteration of the gut microbiota appears to be associated with 'indicator' bacterial taxa, including the family *Lachnospiraceae*. Among those eight species whose populations were decreased following probiotic supplementation and dietary intervention compared with the natural HFD group, one species identified was *B. ovatus*, which was considered to belong to a group of *Bacteroides* species sharing similar phenotypic characteristics to those of *B. fragilis* and *B. vulgatus* that were frequently identified in patients with IBDs, including Crohn's disease and ulcerative colitis (65,66). To a certain extent, probiotic supplementation and dietary intervention may protect an HFD population from suffering from gastrointestinal inflammation disorders, and help to remold a healthy gut bacterial symbiosis through inhibition of IBD-associated specific bacterial taxa. Various bacterial families associated with unclassified species reduced by probiotic and dietary intervention have been identified as *Clostridium* XVIII and *Anaerostipes*. Certain evidence suggests that *Clostridium* XVIII may serve as the next 'smart' probiotics (67), and the genus *Anaerostipes* is associated with potential butyrate-producing bacteria (58). Why these beneficial bacteria are also reduced in number following probiotic intervention remains to be elucidated. A plausible hypothesis explaining this is that the potential of certain probiotic strains to 'rebalance' butyrate concentrations may protect the host under those physiological conditions associated with altered butyrate concentrations (58). One unknown species belonging to the genus *Flavonifractor*, which is capable of cleaving the flavonoid C-ring (68), was determined to decline after combined probiotic and dietary intervention. Depletion of a species belonging to *Flavonifractor* has been demonstrated in obese individuals (69) and another study suggested that treatment with *Bifidobacterium catenulatum* LI10 was able to attenuate this depletion (70), which was not in agreement with the results of the present study. Another study published by Toscano *et al* (71) indicated that, after one month of probiotic intake, a reduction in the population of *Flavonifractor* was observed, which was consistent with the result of the present study. In addition, other unknown species from the genera *Veillonella* and *Fusobacterium*, which may act as pathogens and carcinoma-associated taxa (71-77), were significantly reduced by probiotic and dietary intervention, suggesting an improvement of the gut bacterial community induced by an HFD. Of note, a higher abundance of the supplemented probiotic strains in fecal samples was not observed following supplementation of these probiotics, probably since they did not appear to colonize the intestine themselves within such a short study duration.

In conclusion, the present study revealed that the diversity of gut microbiota was promoted in HFD populations receiving probiotic treatment and dietary intervention, along with an increase in the populations of numerous beneficial species, and a reduction in the number of certain detrimental species. Taken together, the present results suggest that *Lactobacillus acidophilus*, *Bifidobacterium longum* and *Enterococcus faecalis* supplementation and dietary intervention may modulate the gut microbiota, and may provide a natural alternative to treat HFD-associated disorders.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

LMQ, RYG and HLQ designed the study; data collection and analysis was performed by LMQ, RYG and JMH; the manuscript was written by LMQ; and revisions of the manuscript were made by RYG and HLQ; All of the authors provided intellectual input for the study and approved the final version of the manuscript.

Ethics approval and consent to participate

All procedures performed involving human participants were in accordance with the ethical standards of the Institutional and National Research Committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The present study was approved by the Ethics Committee of the Institutional Review Boards of Shanghai Tenth People's Hospital (Shanghai, China) and Jiangyin People's Hospital (Jiangyin, China). Written informed consent was obtained from the patients or their guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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