Food & Function

Red cabbage microgreens modulation of gut microbiota is associated with attenuation of diet-induced obesity risk factors in a mouse model

Journal:	*Food & Function*
Manuscript ID	FO-ART-03-2023-001249.R1
Article Type:	Paper
Date Submitted by the Author:	12-May-2023
Complete List of Authors:	wu, yanbei; Beijing Technology and Business University, School of food and health Pham, Quynhchi ; USDA Agricultural Research Service Wang, Yali; Beijing Technology and Business University Huang, Haiqiu; USDA-ARS BHNRC, Diet Genomics and Immunology Laboratory Jiang, Xiaojing; USDA-ARS BHNRC, Diet Genomics and Immunology Laboratory Li, Robert W; USDA-ARS BARC, Animal Parasitic Diseases Laboratory Yu, Liangli; University of Maryland at College Park, Nutrition and Food Science Luo, Yaguang; U.S. Department of Agriculture, Agricultural Research Service Wang, Jing; Beijing Technology and Business University, Beijing Engineering and Technology Research Center of Food Additive Wang, Thomas T. Y.; USDA-ARS Beltsville Human Nutrition Research Center,

Food & Function

ARTICLE

Red cabbage microgreens modulation of gut microbiota is associated with attenuation of diet-induced obesity risk factors in a mouse model

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

Yanbei Wu [a], Quynhchi Pham [b], Yali Wang [a], Haiqiu Huang [b], Xiaojing Jiang [b], Robert W. Li [c], Liangli Yu [d], Yaguang Luo [e], Jing Wang [a], Thomas T.Y. Wang [b*]

Cruciferous vegetable microgreens, such as red cabbage microgreen (RCMG), are of special interest due to the well documented health promoting effects compared to their mature counterparts. However, little is known of the biological effects of microgreens. The present study used a rodent diet-induced obesity model to investigate the effect of consuming RCMG on the gut microbiota. We found consumption of RCMG exerted profound impacts on microbial composition in mice. Specifically, the species diversity of mice on both low fat (LF) and high fat (HF) diets was significantly increased by consuming RCMG. In comparison to the LF control group, the intake of RCMG increased the gut Firmicutes/Bacteroidetes (F/B) ratio. Furthermore, an unidentified species of the Clostridiales order, increased by RCMG, was found to be negatively correlated with the hepatic cholesterol ester level in mice ($r = -0.43$, $p < 0.05$). In addition, RCMG significantly inhibited HF diet-induced elevation of the genus *AF12*, of which the abundance was positively correlated with body weight gain ($r = 0.52$, $p < 0.01$) and fecal bile acid in mice ($r = 0.59$, $p < 0.01$). Overall, our results demonstrated that consumption of RCMG in the diet can alter the gut microbiota, and attenuation of HF diet-induced body weight gain and altered cholesterol metabolism may be mediated through regulation of the gut microbiota.

Introduction

Obesity is currently one of the most prevalent public health problems in the U.S as well as worldwide.[1,2] Obesity can lead to higher risks for many deadly non-communicable diseases such as diabetes, cardiovascular diseases and cancers, posing serious threats to human health and healthcare burden.[3,4] Epidemiological studies showed that a higher intake of fruits or vegetables, particularly green leafy vegetables, is associated with a significantly reduced risk of obesity and obesity-related diseases.[5,6] However, the beneficial effect of vegetables at the mechanism level remains largely unclear.

Emerging evidence suggests that trillions of bacteria inhabiting the human gastrointestinal tract may act as a bridge between diet and human health.[7,8] It has been reported that the patients with obesity and obesity-related diseases generally suffer from a structural imbalance of gut microbiota, including decreased microbial diversity, changes in the ratio of Firmicutes to Bacteroidetes and reductions in probiotic bacteria such as *Lactobacillus* and *Bifidobacterium*.[9,10] Diet is a critical factor that can rapidly and reversibly affect the structural composition of the gut microbiota. A plant-based diet appeared to generally favour facilitating a healthy gut microbiota and protection against chronic diseases such as obesity.[11] Therefore, identifying food that can promote a healthy microbiome phenotype would be of interest.

Recently, efforts through collaborations between nutritionists, food technology and plant scientists have led to the development of new foods to be tastier, high nutrient density, and rich in health-promoting components. Microgreen's products are a result of such efforts.[12–15] Microgreens are defined as seedlings of edible vegetables usually harvested within 7-14 days of germination, possessing two fully developed cotyledons with or without the emergence of first true leaves.[13] They were first introduced as salad greens for high-end restaurants, and later become popular fresh produce in markets used to enhance taste, texture, color, flavors.[16] The average harvest time of microgreens is later than sprouts and earlier than baby greens, which allows microgreens to have intense flavors, tender textures, vivid colors, and good nutrition.[17] Numerous vegetable species are commercially used for microgreen production, among them is the red cabbage that belongs to the cruciferous vegetable family.[18] Red cabbage microgreen (RCMG) with purplish-green color are rich in vitamins, minerals and phytochemicals, is being considered a good source of nutrients and bioactive substances.[19–21] Studies also showed that the concentrations of phytochemicals such as ascorbic acid, β-carotenoids, phylloquinone and glucoraphanin in RCMG were considerably higher than those reported in their mature plant counterparts.[13] Most of these bio-actives in RCMG have been found to be beneficial to the prevention

[a.] China-Canada Joint Lab of Food Nutrition and Health (Beijing), Beijing Technology &Business University, Beijing 100048, PR China.
[b.] Diet Genomics and Immunology Laboratory, BHNRC, ARS, USDA, Beltsville, MD 20705, USA. E-mail: Tom.Wang@ars.usda.gov.
[c.] Animal Parasitic Diseases Laboratory, BARC, ARS, USDA, Beltsville, MD 20705, USA.
[d.] Department of Nutrition and Food Science, University of Maryland, College Park, MD 20742, USA.
[e.] Food Quality Laboratory, BARC, ARS, USDA, Beltsville, MD 20705, USA.
†Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

of chronic disease and the enhancement of overall health.[17,20,22] Our recent work also indicated that a diet supplemented with RCMG, at a physiologically achievable amount, could significantly lower high fat diet-induced risk factors for health such as weight gain, hypercholesterolemia, and liver inflammation.[17] However, the effect of RCMG on the gut microbiota is still largely unknown.

Based on the existing works in the literature, we hypothesize that microgreens may modulate the gut microbiota and provide a protective effect on obesity-related risk factors. The present study seeks to use RCMG as a model microgreen to address the following questions: 1) will microgreen modulate the gut microbiota? 2) what are the changes? 3) relationship between microbial changes and biological parameter of risk and molecular markers. The current study employs a high fat diet-induced obesity mouse model and a 2 × 2 factorial design (fat levels, with or without RCMG) to test the hypothesis and to elucidate relationships between the health-promoting effects of RCMG and the gut microbiota.

Results

Effects of consuming RCMG for 24 hours on fecal microbiota in mouse

A quantitative PCR-based assay was used to quantify the relative abundance of Firmicutes, Bacteroidetes, *Prevotella*, *Ruminococcus*, *Enterobacteriaceae*, *Bifidobacteria*, *Lactobacillus,* and *Akkermansia muciniphila* in the fecal samples after 24 hours consumption of RCMG to elucidate short-term effects of RCMG on the gut microbiota in mice. As shown in Fig. 1, Firmicutes were significantly increased in feces of mice fed a high-fat diet (HF) compared to a low-fat diet (LF), supplementing RCMG did not affect HF-induced increase in Firmicutes. By contrast, there were no significant differences in Bacteroidetes between the LF group and the HF group, whereas RCMG supplement significantly decrease the abundance of Bacteroidetes in the feces of mice in both the LF and HF groups. Moreover, in comparison with the LF diet, other diet groups exhibit significantly higher Firmicutes to Bacteroidetes ratio in feces. We found that there were no significant differences in *Prevotella, Ruminococcus, Enterobacteriaceae, Bifidobacteria, Lactobacillus, and Akkermansia muciniphila* between the LF and the HF group. The abundance of genera *Prevotella* and *Ruminococcus* was significantly lower in the LFMG group than that in the LF group but there was no difference between HF and HFMG group for the bacteria. There was no difference between the diet groups in *Enterobacteriaceae*. *Bifidobacteria* was lower in the LFMG group compared to the LF group. LF or HF supplemented group as compared to LF and HF, the *Lactobacillus* was lower, and *Akkermansia muciniphila* in LFMG group was lower than LF group.

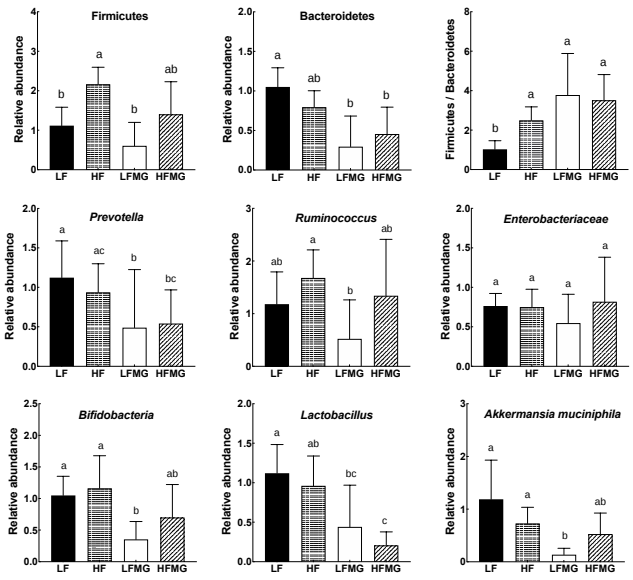

Fig. 1. Effect of RCMG on fecal microbiota in mice fed a low-fat or high-fat diet. The feces were collected from mice 24h after feeding. Bacterial DNA was isolated from feces and quantified with specific primers using qRT-PCR. Results are expressed as the mean ± SD. Columns marked with different letters are significantly different from each other at $p < 0.05$.

Effect of consuming RCMG for 8-weeks on microbial diversity of the cecal microbiota in mouse

To further evaluate the long-term effect of RCMG on the gut microbiota in mice, cecum contents were collected after 8-weeks of feeding for 16S RNA sequencing analysis. The variation in diversity of species among samples (Fig. 2A), measured by Bray-Curtis distance using principal coordinate analysis (PCoA), showed that the animals can be segregated into distinct groups dependent upon the diet consumed ($p < 0.01$). The species composition of the mice on the LF diet was distinct from the mice on the HF diet. Interestingly, the difference in microbiota community between the LF group and the HF group was reduced when the diets were supplemented with RCMG as indicated by overlapping circles between the LFMG and HFMG group. Moreover, the RCMG supplement also had a significant effect on the alpha diversity of the gut microbial community, including both species richness and evenness. As shown in Fig. 2B-E, there were no significant differences for most alpha diversity indices (Chao-1, observed OUT, Shannon) between LF and HF groups. Supplementation of RCMG resulted in significant increases in microbial diversity indices except for Simpson index, regardless of LF diet or HF diet. Chao-1 and observed OTU, two richness-based indices, respectively increased by 7.6% and 10.1% in mice fed LFMG diet compared to mice fed LF diet (Fig. 2B-C). Additionally, Shannon index which was used to characterize species evenness was also enhanced by RCMG (Fig. 2C-D). In the Simpson index, only the LFMG group showed a significant difference from the LF group, there was no difference between the HF and HFMG group. Furthermore, the increase in microbial diversity between the LF and LFMG groups was greater than that between the HF and HFMG groups.

Effect of consuming RCMG for 8-weeks on the composition of cecal microbiota in mouse

The bacterial 16S RNA gene profiling of DNA isolated from cecal luminal contents of mice following 8-weeks RCMG consumption was further used to assess the effect of consuming RCMG on microbial

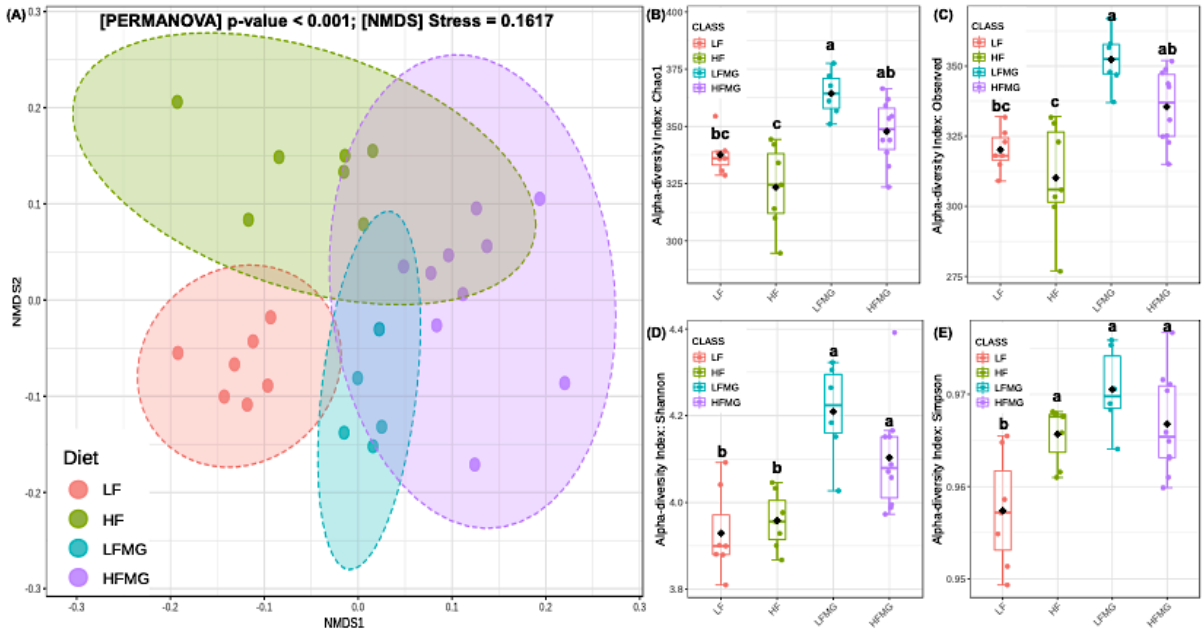

Fig. 2. Effect of RCMG on cecal microbial diversity in mice fed a low-fat or high-fat diet. Cecum content of mice was collected after an 8-week treatment, the bacterial DNA in cecal contents was isolated for 16s rRNA sequencing. (A) Non-metric multidimensional scaling (NMDS) plot for beta diversity based on Bray-Curtis distance matrix at OTU level. The statistical significance of the clustering pattern in 2-D ordination plots was evaluated using Permutational ANOVA (PERMANOVA). (B-E) Boxplots of alpha diversity indices (Chao1, ACE, Shannon, Simpson) at OTU level. Statistical analysis was performed using one-way ANOVA, followed by Tukey's multiple comparisons test. Boxes marked with different letters are significantly different from each other at $p \leqslant 0.05$.

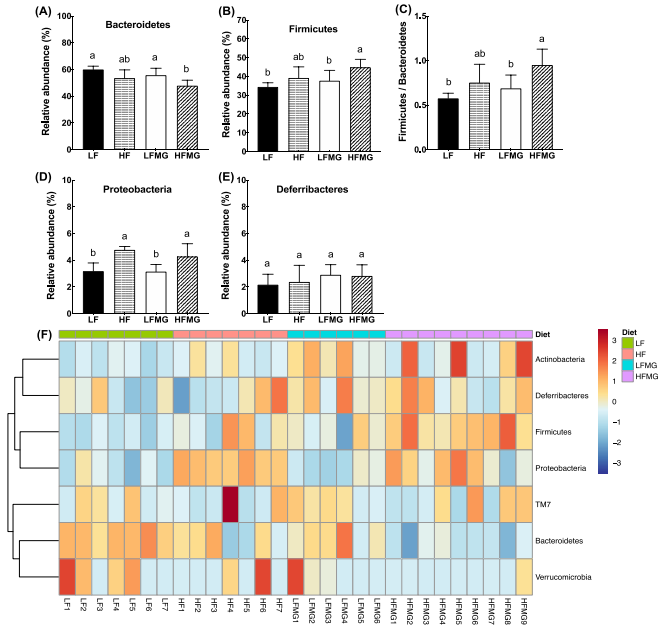

Fig. 3. Alterations of cecal microbiota composition at the phylum level in mice fed different diets. (A, B, D, E) Relative abundance (%) of main phyla in each group, the top 4 phyla shown to account for at least 99% of all OTUs detected for each sample. (C) The ratio of Firmicutes to Bacteroidetes in each group. Results are expressed as the mean ± SD. Bars marked with different letters are significantly different from each other at $p \leqslant 0.05$. (F) Heatmap of the relative abundance of 16S rRNA gene sequences taxonomically classified to phylum level. The hierarchical clustering analysis was performed by Microbiome Analyst using Ward clustering algorithm and Euclidean distance measure.

community structure. A total of 7 phyla were identified from all samples, the most abundant phylum detected in the cecum of mice was Bacteroidetes (60%), followed by Firmicutes (34%), *Proteobacteria* (3%), and *Deferribacteres* (2%), accounting for more than 99% of the bacterial sequences (Table S2). At the phylum level, consumption of RCMG didn't significantly influence the dominant phyla of mice. For example, mice fed HFMG diet had lower abundance of Bacteroidetes and higher abundance of Firmicutes than mice fed LF or LFMG diet, while these two dominant phyla had no significant difference between HF- and HFMG-fed mice (Fig 3A, B, F). Correspondingly, the Firmicutes to Bacteroidetes ratio (F/B ratio) of HFMG group was significantly increased compared with LF group and LFMG group but was not significantly different from those of HF group (Fig. 3C). In addition, HF- and HFMG-fed mice had higher abundance of *Proteobacteria* than of LF- or LFMG-fed mice, and there was no significant difference between HF and HFMG diet group. The relative abundance of *Deferribacteres* was not significantly different among the four diet groups (Fig. 3D, E, F).

At the family level, more than 65 bacterial taxa were detected. Fig. 4A showed the top 15 families which covered more than 99% of the bacterial sequences including two unclassified families in order *Clostridiales* and order *RF32* (Fig. 4A). Among the top 15 families, the relative abundance of five families *Ruminococcaceae*, *S24-7*, unclassified *Clostridiale*was, *Desulfovibrionaceae* and *Dehalobacteriaceae* were affected by different diets (Fig. 4B-F). Compared to the LF diet group, the HF diet group has a higher abundance of *Ruminococcaceae* and *Desulfovibrionaceae* and a lower abundance of *S24-7*. Supplementing RCMG in LF diet led to a higher abundance of *Desulfovibrionaceae* but a lower abundance of *S24-7* and *Dehalobacteriaceae* compared to the LF diet group. The

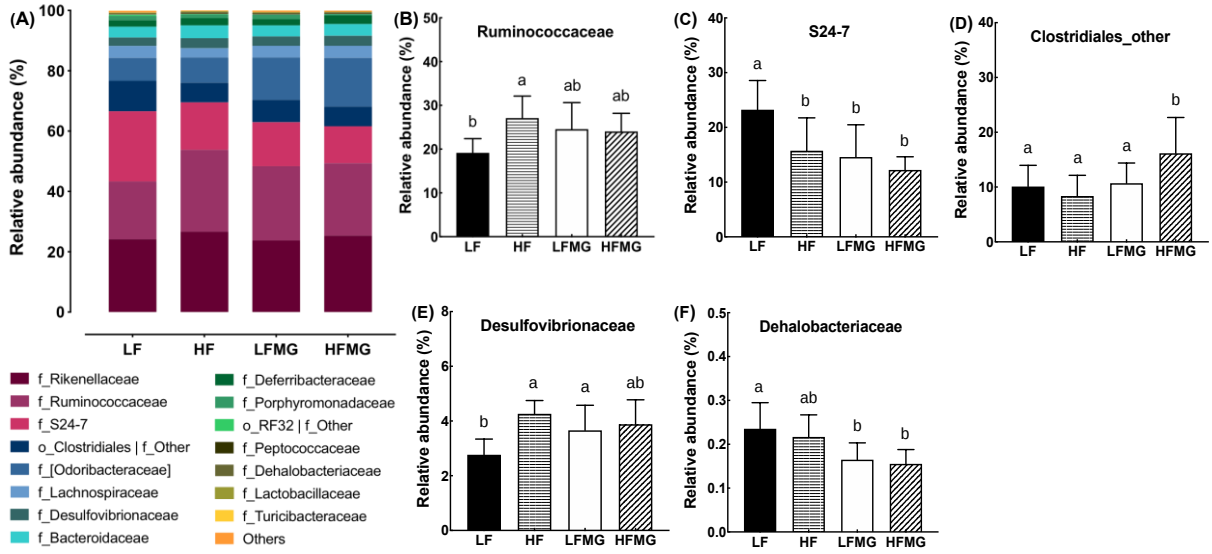

Fig. 4. (A) Alterations of cecal microbiota composition at the family level in mice fed different diets. The top 15 families are presented, whereas the sum of the remaining taxa is displayed as "Others". (B-F) Comparison of the relative abundance at the family level among different groups. Results are expressed as the mean ± SD. Bars marked with different letters are significantly different from each other at $p \leqslant 0.05$.

consumption of RCMG appears not to affect the HF diet-induced changes of gut microbiota at a family level. However, it is worth noting that an unclassified *Clostridiales*, a member of Firmicutes, was specifically higher in mice fed HFMG diet as compared to the other diet groups.

Identification of microbial biomarkers associated with a diet

To identify taxonomic differences in the microbial community of mice fed on different diets for potential biomarker discovery, linear discriminant analysis effect size (LEfSe) method was applied at the genus level. As shown in Fig. 5A, a total of 42 differentially abundant bacterial taxa were identified in four diet groups, of which 15 genera with LDA score (log10) > 3 were assigned as potential biomarkers with statistical and biological significance (Table 1), and 3 genera including *AF12*, *Bilophila* and *Lactococcus* remained significantly different between diet groups at false discovery rate (FDR) adjusted $p < 0.05$ (Fig. 5C). The relative abundance of genus as microbial biomarkers was significantly higher in the corresponding group than in the other three groups. Specifically, the most discriminative genera among the diet groups were unclassified *S24-7*, *Parabacteroides*, unclassified *Mogibacteriaceae*, *Dehalobacterium* and *Lactococcus* in LF group, *AF12*, *Oscillospira,* unclassified *Ruminococcaceae* and *Bilophila* in HF group, unclassified *Erysipelotrichaceae* and unclassified *Clostridiaceae* in LFMG group, and *Clostridiales*, *Coprococcus*, *Desulfovibrio* and *Adlercreutzia* in HFMG group, respectively (Table 1).

In addition, the random forest analysis was conducted to dissect the relationships between microbial taxa and dietary treatments, identifying 8 most important genera by LEfSe (Fig. 6 A). The genus *AF12*, a possible microbial biomarker of HF group, was ranked among the most important features based on mean decrease accuracy and was found to be significantly lower in HFMG group than HF group. Furthermore, the consumption of RCMG causes a reduction of *Parabacteroides* and an increase of unclassified *Clostridiales* in both LF and HF groups. (Table S2 and Fig. 6 B). These results suggested that the increase of unclassified *Clostridiales* induced are important features discriminating HF diet group from HFMG group and may have a critical relevance to physiological functions of mice. Additionally, two important genera, unclassified *Clostridiales* and *Lactococcus*, were also identified as a microbial signature with a high accuracy (AUC=0.939) for the predictive of RCMG supplementation status by using selbal analysis. As shown in Figure 5C, the identified microbial signature given by the log ratio of the abundance of unclassified *Clostridiales* (denominator) and *Lactococcus* (numerator) was a negative value, indicating that unclassified *Clostridiales* had a much higher abundance than that in *Lactococcus*. RCMG supplemented groups are associated with a lower balance value, that is, larger relative abundance of unclassified *Clostridiales* than of *Lactococcus*.

Relationship between gut microbial communities and biochemical indexes of mice

The top 15 genera accounting for ~98% of the bacterial sequences were selected to represent the gut microbial communities for Pearson correlation analysis (Table S3) with diet-induced obesity risk factors including body weight gain, plasma lipoprotein levels, and hypercholesterolemia. According to the results in Table S3 and illustrated in Fig. 7, several important bacterial genera including unclassified *S24-7*, unclassified *Clostridiales*, unclassified

Table 1. Relative abundance (%) of main biomarkers identified by LEfSE analysis. [a,b]

Phylum/ Class; Order; Family	Genus	Diet			
		LF	HF	LFMG	HFMG
Bacteroidetes					
Bacteroidia; Bacteroidales; Rikenellaceae	***AF12***	4.08±0.62[a]	7.29±1.33[b]	3.99±1.11[a]	5.18±1.51[a]
Bacteroidia; Bacteroidales; S24-7	Other	23.24±5.32[a]	15.74±6.01[b]	19.75±2.62[a]	12.12±2.29[b]
Bacteroidia; Bacteroidales; Porphyromonadaceae	*Parabacteroides*	1.41±1.39[a]	1.25±1.67[ab]	0.45±0.32[b]	0.47±0.65[b]
Firmicutes					
Clostridia; Clostridiales; Ruminococcaceae	*Oscillospira*	10.17±1.97[a]	14.01±2.74[b]	9.81±2.19[a]	12.57±3.04[ab]
Clostridia; Clostridiales; Ruminococcaceae	Other	5.17±1.52[a]	8.70±1.80[b]	5.33±1.51[a]	7.55±1.44[b]
Clostridia; Clostridiales; Other	**Other**	10.10±3.87[ab]	8.36±3.78[a]	14.17±2.82[ab]	16.23±6.17[b]
Clostridia; Clostridiales; Lachnospiraceae	*Coprococcus*	0.24±0.05[a]	0.34±0.26[ab]	0.36±0.13[ab]	0.53±0.25[b]
Clostridia; Clostridiales; Mogibacteriaceae	Other	0.04±0.01[a]	0.02±0.02[a]	0.03±0.01[a]	0.02±0.01[a]
Clostridia; Clostridiales; Dehalobacteriaceae	*Dehalobacterium*	0.24±0.06[a]	0.22±0.05[ab]	0.18±0.1[ab]	0.16±0.03[b]
Bacilli; Lactobacillales; Streptococcaceae	*Lactococcus*	0.05±0.01[a]	0.04±0.01[a]	0.03±0.01[a]	0.03±0.01[a]
Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae	Other	0.01±0.01[a]	0.00±0.00[ab]	0.02±0.02[a]	0.00±0.00[b]
Clostridia; Clostridiales; Clostridiaceae	Other	0.01±0.00[a]	0.00±0.00[a]	0.01±0.01[a]	0.00±0.00[b]
Proteobacteria					
Deltaproteobacteria; Desulfovibrionales; Desulfovibrionaceae	*Bilophila*	2.52±0.60[a]	4.03 ±0.47[b]	2.43±0.56[a]	3.59±0.85[b]
Deltaproteobacteria; Desulfovibrionales; Desulfovibrionaceae	*Desulfovibrio*	0.25±0.10[a]	0.23±0.09[a]	0.24±0.05[a]	0.32±0.08[a]
Actinobacteria					
Coriobacteriia; Coriobacteriales; Coriobacteriaceae	*Adlercreutzia*	0.01±0.00[a]	0.01±0.01[a]	0.02±0.01[a]	0.02±0.01[a]

[a] The grey highlight shows the abundance of biomarker assigned to the corresponding group. [b] Tukey's multiple comparisons test was used to characterize the difference between the groups. Results are expressed as mean ± SD and marked with different letters which indicate significantly different from each other at $p \leq 0.05$)

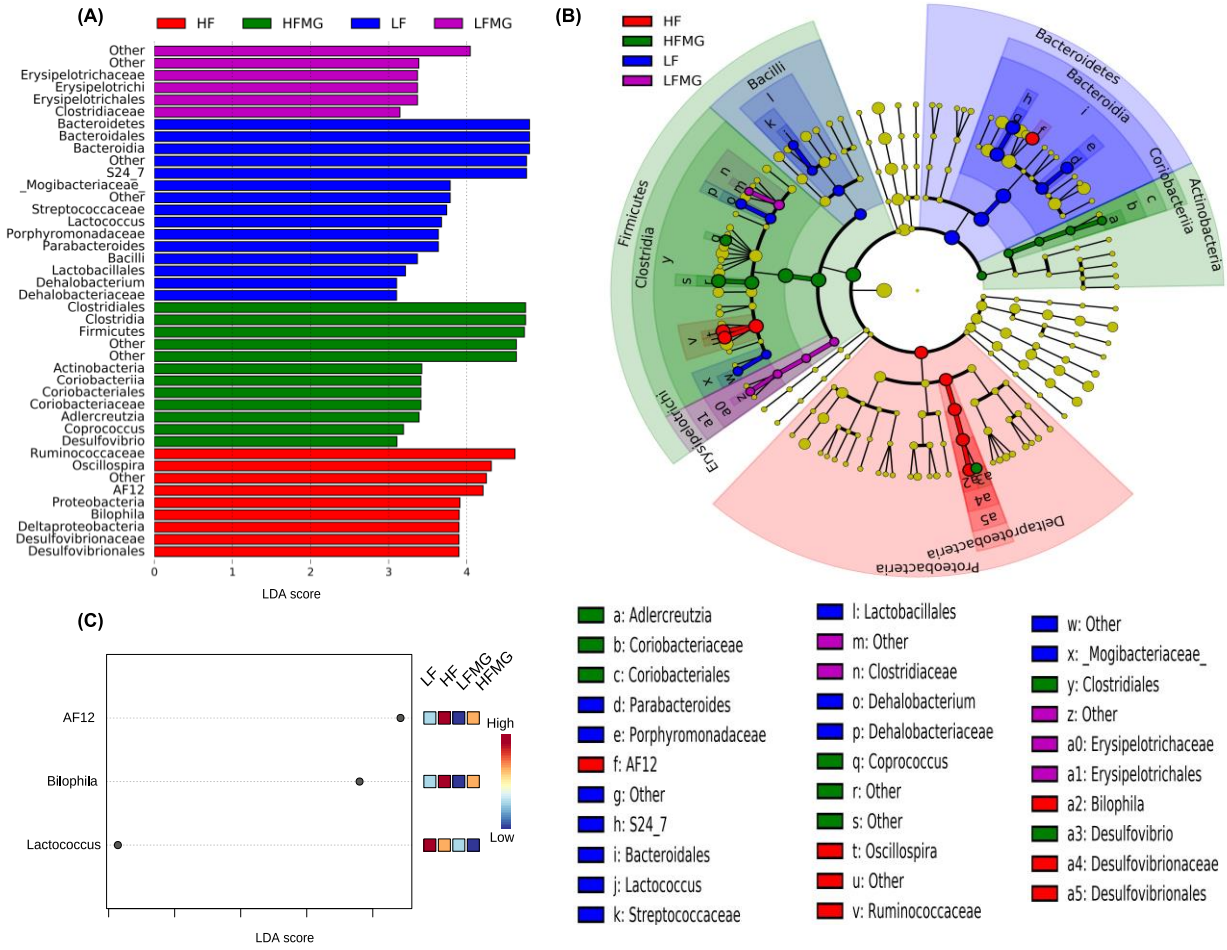

Fig. 5. Comparison of cecal microbiota variation at the genus level using LEfSe analysis. (A) Histogram of the LDA scores for differentially abundant features among four groups (Only the taxa with a significant LDA score (log10) > 3 are shown); (B) Taxonomic cladogram of differentially abundant features represented by rings with phyla in the outermost ring and genera in the innermost ring. The diameter of the circle is proportional to the relative abundance and the circle color corresponds to the individual dietary treatment in which the taxon is the most abundant among four groups; (C) The differentially abundant features with FDR-adjusted $p < 0.05$.

Ruminococcaceae, *AF12*, *Bilophila* and unclassified *RF32* are associated with biochemical/physical parameters. An unclassified genus in family *S24-7* was negatively associated with body weight gain and low-density lipoprotein levels (LDL), while *AF12* showed positive correlations with these two parameters and fecal bile acid. The abundance of an unclassified genus in order *Clostridiales* was negatively associated with hepatic cholesterol ester which positively associated with an unclassified genus in family RF32. An unclassified genus in family *Ruminococcaceae* and *Bilophila* were positively associated with body weight gain and fecal bile acid. Moreover, it is observed that the selected top 15 abundant genera had no correlation to HDL and Hepatic triglycerides.

Effect of RCMG on the gut microbial ecological network

The microbial ecological network, a group of OTUs that are highly connected among themselves, defined as modules, were elucidated using Random matrix theory (RMT)-based approach. We found dietary supplementation with different interventions affected the interactions between the members of the microbial community. As shown in Table S4 and Fig. 8, the LF group's network consisted of 23 modules with 247 nodes and 416 lines, while the HF group's network contained 23 modules with 245 nodes and 483 lines. After dietary intervention by RCMG, the network for LF diet contained 29 modules with 302 nodes and 643 lines, while the network for HF diet consisted of 29 modules with 257 nodes and 471 lines. This suggests the dietary supplementation with RCMG increased the number of nodes (network size) within the ecological network. For the LF, HF, LFMG and HFMG groups, there were 12, 10, 12 and 10 modules with more than 5 nodes, respectively. Moreover, the dominant interactions in the four networks were positive interaction. Also, it can be seen from Table 4 that the LF and HF networks showed similar avgCC value, which was lower than that of LFMG network and higher than that of HFMG network, indicating that these networks are naturally different functional units. When the power law model was used to fit the network connectivity distribution curves, the $R^2 > 0.8$ indicated that HFMG network was a scale-free network. Compared with the LFMG network, the HFMG network showed a lower avgK value, suggesting a lower network complexity, whereas, it exhibited lower avgCC value and M value, indicating lower modularity.

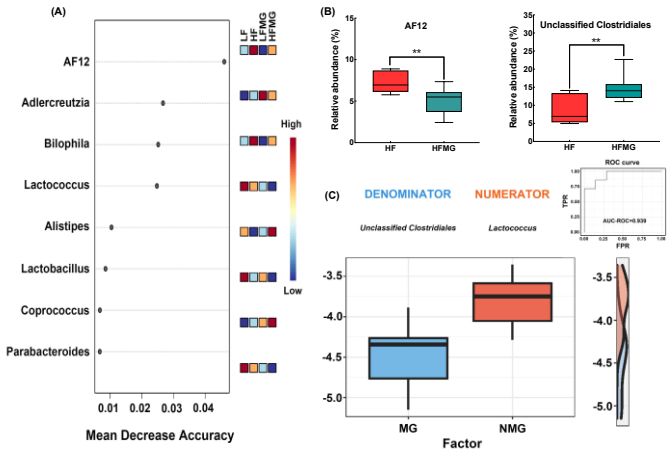

Fig. 6. Important genus related to the consumption of RCMG (MG). (A) The top 8 important genera identified by random forest analysis from top to bottom based on Mean Decrease Accuracy. The genera are ranked by their relative importance to classification accuracy, a higher value of mean decrease accuracy indicates the importance of that genus in predicting group. (B) Intake of RCMG had a profound effect on genus *AF12* and unclassified genus in family *Clostridiales*. Results are expressed as the mean ± SD, ** indicated $p \leq 0.01$. (C) Microbial signatures at genus level selected by selbal analysis based on balance score to discriminate different treatments (MG: supplementation of RCMG; NMG: non-supplementation of RCMG) under a high-fat diet. The box plots represent the distribution of the balance values for each group. The right (vertical) panel of the figure represents the ROC curves with the AUC values (top) and density curves (bottom) for each category.

Nodes/OTUs in a module play different topological roles in the network. To identify some key nodes in the network, plots were generated based on within-module connectivity (Zi) and among-module connectivity (Pi). As shown in Fig. S1, the majority of OTUs that were identified in the LF, HF, LFMG and HFMG groups were peripherals. Several nodes acting as connectors that linked different modules together were identified in scatter plots for the LF, HF and LFMG groups. Notably, in the LF group, six OTUs (OTU380534, OTU1108453, OTU341713, OTU387615, OTU340853 and OTU1517779), respectively belonging to the genera *S24-7*, *Clostridiales*, *Ruminococcaceae*, *Oscillospira*, *Lachnospiraceae*, *Rikenellaceae*, were identified as connectors, and an OTU of *S24-7* (OTU390633) served as module hub. In the HF group, four OTUs (OTU2315700, OTU404691, OTU264657 and OTU346804), respectively belonging to the genera Ruminococcus, *S24-7* and *Ruminococcaceae*, played as connectors, and an OTU of *Lachnospiraceae* (OTU340853) was identified as a module hub. In the LFMG group, four OTUs (OTU2797565, OTU259884, OTU208571 and OTU215495), respectively belonging to the genera *Mogibacteriaceae*, *Oscillospira*, *Coprococcus* and *S24-7*, were identified as connectors, and an OTU of *Clostridiales* (OTU400599) served as a module hub. In the HFMG group, no OTUs were identified as connectors, and two OTUs from *Oscillospira* (OTU397363) and *Clostridiales* (OTU197853) served as module hubs, respectively. The above-mentioned genera acting as a connector species can link two or more different modules together. As well-acknowledged, network

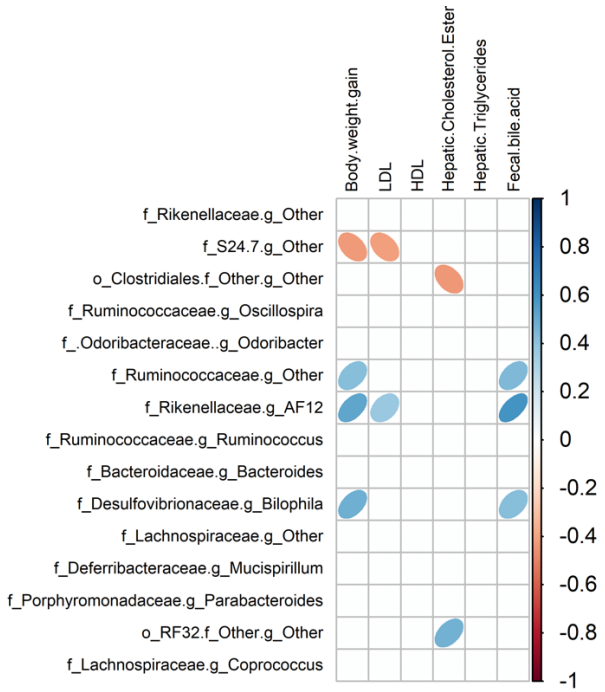

Fig. 7. Pearson's correlation matrix of physiological parameters affected by diet and the top 15 abundant genera. Results were corrected for multiple hypothesis testing using the Benjamini-Hochberg correlation method. Genera are listed from the most abundant to the least. Blue ellipses represent positive correlation, while red ellipses represent the negative correlation.

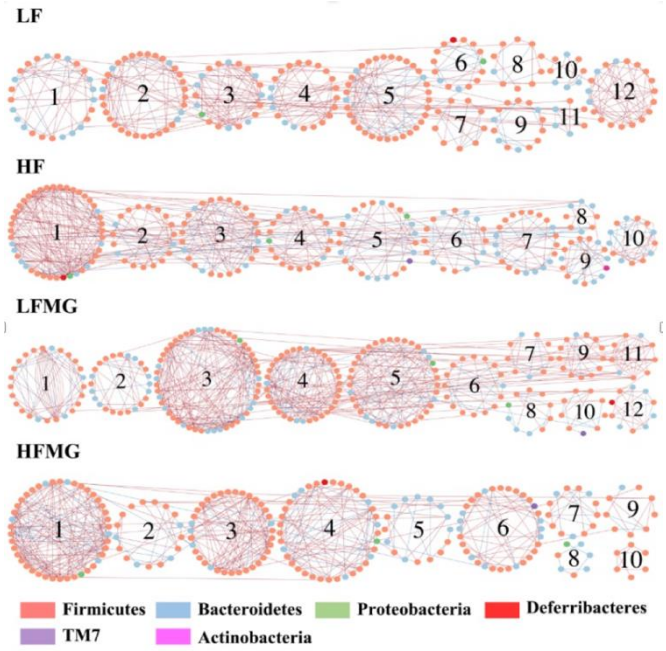

Fig. 8. The molecular ecological networks of the intestinal microbiota in different diet groups (LF: a low-fat diet, HF: a high-fat diet, LFMG: a low-fat diet supplemented with RCMG powder, HFMG: a high-fat diet supplemented with RCMG powder). Each node represents an OTU. Colors of the nodes indicate different major phyla. A red line indicates a positive interaction between two individual nodes, while a blue line indicates a negative interaction.

hubs, the nodes with a very high between-module and among-module connectivity value, is supposed to be important for the coherence of the global network and as well as its own module. They can be considered as the keystone species in a microbial community. However, that there were no network hubs identified in each of the four global networks analyzed in this work (Fig. S1).

Table 2 listed the correlations between node connectivity and OTU significance with physiological traits in microbial co-occurrence networks to elucidate the involvement of specific bacterial genus on biological function. *Bacteroidaceae* (Family), *Bacteroides* (Genus) appeared to be involved in cholesterol metabolism (bile acid, hepatic free cholesterol) in the LF, HF, and LFMG diet groups. The supplementation of red cabbage microgreen appeared to recruit additional bacteria to be involved in cholesterol metabolism in the LFMG diet group. *Rikenellaceae* (Family) was found to be associated with body weight. *Ruminococcus* (Genus) and Gnavus (Species) were identified to be associated with fecal bile acid. Interestingly, unlike LFMG, in the HFMG group, only *Coprococcus* (Genus) and *Rikenellaceae* (Family) were found to be associated with body weight and hepatic triglycerides.

The relationship between individual modules and physiological traits were further evaluated (Table 3). Each diet appeared to have a different set of networks associated with physiological traits. Under LF diet, at least 3 modules were positively correlated with body weight, hepatic free cholesterol and fecal bile acid, and 1 module was negatively correlated with hepatic cholesterol ester. However, the correlations between modules and physiological traits in LF group were drastically changed after the dietary treatment with red cabbage microgreen on the LF diet. There is a module positively related to LDL levels and 2 modules negatively related to body weight and HDL levels in LFMG group. Under the HF diet, 2 modules were negatively related to body weight and Hepatic cholesterol ester and 4 modules were positively associated to VLDL levels, hepatic free cholesterol and fecal bile acid. HFMG group also had modules positively and negatively correlated with body weight and fecal bile acid, respectively, while also had more modules positively associated with liver cholesterol and triacylglycerol levels in comparison to the HF group.

Table 2. Correlations between node connectivity and OUT significance of physiological traits in microbial co-occurrence networks.

Diet group	Physiological traits	Bacterial taxa (rank)	r[a]	*p*-Value[b]
LF	Fecal bile acid	*Bacteroides* (Genus)	0.741	0.002
HF	Hepatic free cholesterol	*Bacteroides* (Genus)	0.684	0.001
LFMG	Body weight	*Ruminococcus* (Genus)	0.413	0.019
	LDL	*Bacteroides* (Genus)	0.716	0.008
	Fecal bile acid	*Gnavus* (Species)	0.600	0.037
HFMG	Body weight	*Coprococcus* (Genus)	0.721	0.01
	Hepatic triglycerides	*Rikenellaceae* (Family)	0.743	0.048

[a] Correlation coefficient based on the Mantel test. [b] The significance (probability) of the Mantel test.

Table 3. Correlations between module eigengene and physiological traits in microbial co-occurrence networks.

Diet group	Module	Physiological traits	r[a]	p-Value[b]
LF	9	Body weight	0.84	0.02
	7	Hepatic cholesterol ester	0.88	0.008
	7	Hepatic free cholesterol	-0.92	0.003
	1	Fecal bile acid	0.81	0.03
HF	10	Body weight	-0.81	0.03
	2	VLDL	0.83	0.02
	9	VLDL	0.89	0.007
	8	Hepatic cholesterol ester	-0.79	0.04
	6	Hepatic free cholesterol	0.84	0.02
	7	Fecal bile acid	0.79	0.03
LFMG	11	Body weight	-0.99	0.001
	6	LDL	0.97	0.007
	5	HDL	-0.99	0.0006
HFMG	5	Body weight	-0.79	0.01
	8	Hepatic cholesterol ester	0.71	0.03
	1	Hepatic free cholesterol	0.72	0.03
	2	Hepatic triglycerides	0.68	0.04
	6	Hepatic triglycerides	0.72	0.02
	3	Hepatic triglycerides	-0.84	0.0054
	4	Fecal bile acid	0.68	0.04

[a] Correlation coefficient based on the Mantel test. [b] The significance (probability) of the Mantel test.

Discussion

The current study confirmed our hypothesis that the consumption of microgreens can modulate the gut microbiome. Several unique gut microbiome features were also identified between the diet groups in the study. Additionally, we also reported the association of specific bacteria genera with risks factors associated with consumption of a high-fat diet.

Our PCR analysis of fecal bacteria 24 hours after consumption of microgreen generally agrees with the literature that changes to the main composition of fecal microbiota can occur relatively quickly.[23] The ability to adapt to a diet change appeared to be quick but maybe more transient, as in some cases, the differences disappeared after 8 weeks.[24] We also observed the fecal color changes from very

colorful then back to similar dark brown for all mice on different diets, suggesting that adaptation occurred. Up-regulation of metabolic enzymes in bacteria or growth of bacterial populations may allow for a more efficient process of compounds that contributed to the color, such as anthocyanin and chlorophyll, etc.[25,26] These types of adaptation may have physiological consequences. Phytochemicals are known to be catabolized by intestinal bacteria and allowed for absorption into the host. Therefore, depending on whether a compound is partially processed or completely metabolized, one may reason that there may be more or less phytochemical being absorbed.[27,28] Consider the potential health impact of some of the health-promoting phytochemicals, this process may influence the bioavailability and consequently biological efficacies of a compound. However, more work is needed to validate these hypotheses.

The PCoA result indicated that LF and HF groups are very different in terms of the cecal bacterial diversity. The introduction of RCMG into the respective diet seemed to even out the difference between the two diets as indicated by the increased overlaps between the animal's gut microbiotas. Additionally, adding RCMG into the LF or HF diet also modulated alpha diversity except on the Simpson index. Overall, adding RCMG into a diet would increase the diversity of the gut microbiome. It has been reported that low microbial diversity is associated with adiposity and dyslipidemia in overweight/obese humans.[29] We reason that RCMG may prevent high-fat diet induced increase in weight gain by significantly improving the cecal microbial richness.

The composition difference of the gut microbiome at the phylum levels appeared relatively stable and the addition of RCMG appeared not overtly affect the composition. However, RCMG appeared to affect specific bacterial families, such as *S24-7*, *Desulfovibrionaceae, Dehalobacteriaceae.* It is worth noting that the diet matrix may play a role in the phenotypic display of these changes as they appeared to be significant in the LF diet matrix but not in the HF diet matrix. To further support this notion, the unclassified *Clostridiales* were significantly higher only in the HFMG group. Therefore, the effects of food on the gut microbiome composition may depend on other foods in the diet matrix and may be quite complicated.

The results from microbial network analysis indicated that, although the microbial network composition was different between the four diet groups, the proportion of nodes (OTUs) at phylum level in each network did not differ significantly. However, the topological roles of nodes in each network were completely distinct, which likely reflected habitat heterogeneity or trophic specialization under different diet.[30]

LEfSe and selbal analysis provide microbial signatures information for different dietary treatments that can be developed as biomarkers for prediction of treatment outcomes. However, there appeared to be no overlapping markers especially for those diets supplemented with RCMG. These data provide additional support that matrix may play a role in phenotypic display of a specific food. Interestingly, paired comparisons, such as those illustrated in Figure 6 provide some useful information. *AF12* appeared to be one biomarker that allows for differentiation of HF diet from HFMG diet in our study. *AF12* is known to be a genus found to be abundant in obese mice than lean mice,[31,32] hence it may be considered as a candidate for dietary risk marker. Also, unclassified *Clostridiales* and *Lactococcus* appeared to have a relatively high discriminative power for the RCMG feeding under the paired comparison condition and may be a candidate marker for RCMG intake. Further studies are necessary to validate these hypotheses.

The alteration of gut microbiota has been considered as a critical contributor to increasing risk of various chronic diseases, such as obesity, diabetes and inflammatory bowel disease.[33] In this study, several genera were identified to be associated with the diet induced obesity risk factors. *AF12* genus, identified as a biomarker in HF group, was positively correlated with body weight gain, the amount of LDL and fecal bile acid in mice. The increased abundance of *AF12* by HF diet was significantly attenuated by supplementation of RCMG. Also, the unclassified *Clostridiales* order, significantly increased by RCMG, was found to be negatively correlated with the hepatic cholesterol ester level in mice. These characteristics may be further explored for dietary risk/intake assessment.

The correlation analysis in microbial co-occurrence network further revealed the relationships between network topologies and physiological traits. A group of bacterial taxa was found in diet-specific microbial networks to be highly associated with different physiological traits, in our case cholesterol metabolism. In particular, hepatic cholesterol, bile acid as well as hepatic triglyceride were associated with the OTUs from genus *Bacteroides* in both LF and HF diet groups. It is likely that these bacteria may be related to dietary fat consumption and therefore cholesterol, bile acid metabolisms. The presence of RCMG appears to introduce additional bacteria into overall metabolism, as bacteria associated with cholesterol metabolism other than *Bacteroides* were found in the LFMG group. Again, the diet matrix appeared to play a role in the phenotypic display, the OTUs associated with physiological traits are quite different in the HFMG group compared to LFMG group. The notion also supported by the correlation analysis between module-based eigengenes and physiological traits in microbial co-occurrence network. The pMEN of four experimental groups all showed close correlations between submodules and physiological parameters, but the responses of different submodules in the same pMEN to physiological parameters were significantly different. Overall, although one can say RCMG can alter microbial interaction network, the matrix may influence what networks were affected. Of note, it is in the LFMG group that several bacteria were found to be associated with body weight. Our previous studies indicate the LFMG group tends to have a higher growth rate than the LF group. This effect of MG on body weight may be due to modulation of the gut microbiome. Although exact mechanisms of how the gut microbiome act remains to be elucidated.

Conclusions

In summary, we confirmed the hypothesis that RCMG can modulate the gut microbiota. The effect of RCMG can be on diversity, composition as well as microbial co-occurrence network. Our study also highlights specific bacteria that may be a biomarker for a diet, such as for the HF diet and the unclassified *Clostridiales* for RCMG diet. We propose that the interaction between food in the diet matrix may influence the phenotypic display of effects on the gut microbiome exerted by a food and a critical factor to consider.

Experimental

Animal and diets

Male C57BL/6NCr mice, (aged 5 weeks, approximately 20 g), were purchased from Charles River Laboratories (National Cancer Institute, Frederick, MD, USA), and were single-housed in filter-top cages at the Beltsville Human Nutrition Center's animal facility with a 12:12 h light-dark cycle. Before the experiment, mice were acclimatized to the environment by feeding a standard rodent chow for 1 week. After that, a total of 40 mice were randomized into four experimental groups (10 mice/group) with four different diets: (1) low-fat diet (LF) (10 kcal% fat diet); (2) low-fat diet supplemented with RCMG powder (LFMG) (10 kcal% fat diet containing 10.9 g/kg diet); (3) high-fat diet (HF) (45 kcal% fat diet); (4) high-fat diet supplemented with RCMG powder (HFMG) (45 kcal% fat diet containing 10.9 g/kg diet). Mice (10 per group) had free access to water and the corresponding diet for 8 weeks. Food intake and body weight were recorded weekly. The experimental diets were processed and provided by Research Diets (New Brunswick, NJ, USA) with low-fat diets consisted of 70% carbohydrate, 20% protein, and 10% fat on a caloric basis, and high-fat diets of 35% carbohydrate, 20% protein and 45% fat on a caloric basis. RCMGs samples were added into diets in the form of dry powder, and the amount of supplementation in the mice's diet was calculated based on the equivalence of 200 g of vegetables/day/person (60 kg) according to the Dose Translation Formula. All animal experiments were conducted under an animal study protocol (Protocol 14-006) that was reviewed and approved by the USDA, ARS, Beltsville Area Institutional Animal Care and Use Committee (IACUC).

Fecal DNA extraction and quantitative PCR analysis

Fecal samples were collected from mice 24 h after treatment and then homogenized with Precellys (Bertin Technologies, France) at 7500 rpm for 1 min. Bacterial DNA was extracted from the feces by using a QIAamp DNA Stool Mini Kit from Qiagen according to the manufacturer's protocol with modification. The DNA was eluted from the column with 100 µL nuclease-free water. The DNA concentration of the elution was determined using absorbance at 260 nm, followed by serial dilutions to the final concentration of 10 ng/µL. Relative levels of major bacterial species were quantified by real-time PCR with a reaction system of 10 µl SYBR® Green Real-Time PCR Master Mix, 0.25 µL 500 nM custom-made oligo primers, 4.5 µL water, and 5 µL DNA. The real-time PCR experiments were performed on 7900T Real-Time PCR System (Applied Biosystems™, Forest City, CA, USA) and the specific primers used in this study are shown in Table S1.[34,35]

Cecal DNA extraction and 16S rRNA sequencing

Cecal samples were collected when mice were sacrificed and quickly frozen in liquid nitrogen, then stored at -80 °C until needed. Total DNA was isolated from cecal samples following the manufacturer's instructions of QIAamp Fast DNA Stool Mini Kit (Qiagen, USA). The purity and concentration of the DNA obtained were determined using Nanodrop 8000 spectrophotometer (Thermo Scientific, Wilmington, DE). The hypervariable V3-V4 regions of the 16S rRNA gene were amplified as previously described. The PCR primers were as follows: forward primer, 341/357F, CCTACGGGNGGCWGCAG; reverse primer, 805/785R: GACTACHVGGGTATCTAATCC. A total of 20 cycles of PCR amplification was conducted. The amplified products were purified using Agencourt AMPure XP bead kits (Beckman Coulter, USA), and then quantified using a High Sensitivity DNA Kit (Agilent, USA). The purified amplicons from individual samples were pooled based on an equal molar ratio and their respective samples-specific barcodes. The library pool was sequenced by using an Illumina MiSeq Reagent Kit v3 and Illumina MiSeq sequencer (Illumina, USA) as described previously.[36,37]

Sequence data analysis

The sequence data were preprocessed using MiSeq Control Software version 2.4.1. The quality of raw sequences was checked using Fast QC (version 0.11.2). The low-quality reads and the four maximally degenerate bases ("NNNN") at the most 5' end of the read pairs was removed using Trimmomatic (version 0.36). The processed paired-end reads were then merged using *join_ paired_ends.py*. The parameter used were set as follows: the minimum overlap length was 20 bp and the maximum allowed mismatches within the overlapping region was 5%. The sequencing data were processed with the Quantitative Insights into Microbial Ecology pipeline (QIIME, version1.9.1). The "closed reference" pipeline (*pick_closed_reference_otus.py)* was used for picking operation taxonomic unit (OTU), and the taxonomy assignment was based on the GreenGene database (version 13.8). Then, PICRUSt (v1.1.3) was used to predict gene contents and metagenomic function information with the OTU table based on annotated genes in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database2.

Statistical analysis and visualization

Species diversity assessment, hierarchical clustering, heatmap visualization, and random forest analysis were performed using the MicrobiomeAnalyst platform (http://www.microbiomeanalyst.ca). The significantly different features (taxa) between four experimental groups were identified using an online galaxy tool (http://huttenhower.sph.harvard.edu/galaxy/) based on a linear discriminant analysis (LDA) effect size (LEfSe) algorithm. Microbial signatures or balances were identified using selbal (R version 3.6.1) with default parameters.[38] Correlations between changes in relative abundance at genus level and changes in obesity-related risk factors, including weight gain, plasma lipoprotein, and lipid metabolites, were calculated using GraphPad Prism 8 with two-tailed Pearson's correlation or Spearman's rank methods depending on the distribution of the data. The selection of physiological data was based on our previous study.[17] Comparisons of taxon relative abundances between the experimental groups were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). The data were expressed as means ± SD and analyzed using one-way ANOVA followed by Tukey's post hoc test. All results were considered statistically significant at $p < 0.05$.

Microbial co-occurrence networks were constructed following a random matrix theory (RMT)-based pipeline described by Zhou et al.[39] The OTUs that detected in less than 50% of all samples were excluded for further analysis. A fast-greedy modularity optimization procedure was used for module separation. The within-module degree (Z) and among-module connectivity (P) were calculated and plotted to present topological roles of individual nodes in each

network. A Mantel test was performed to measure the relationship of the network topology and physiological traits by calculating OTU significance and node connectivity. The relationships between modules and physiological traits were determined using Pearson correlation analysis. The network was visualized using Cytoscape v3.6.1.[17]

Author Contributions

Yanbei Wu: Investigation, writing – original draft; **Quynhchi Pham**: Investigation, and review & editing; **Yali Wang**: Investigation; **Haiqiu Huang**: Investigation; **Xiaojing Jiang**: Investigation; **Robert W. Li**: Investigation, and review & editing; **Liangli Yu**: Review & editing; **Yaguang Luo**: Review & editing; **Jing Wang**: Review & editing; **Thomas T.Y. Wang**: Conceptualization, supervision, and review & editing.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This research was funded by USDA appropriated fund #8040-51530-057-000D (TW) and the National Natural Science Foundation of China #32101950 (YW).

Notes and references

1 S. Rössner, *Int. J. Obes.*, 2002, **26**, S2–S4.
2 A. C. Skinner, S. N. Ravanbakht, J. A. Skelton, E. M. Perrin and S. C. Armstrong, *Pediatrics*.
3 A. Must, J. Spadano, E. H. Coakley, A. E. Field, G. Colditz and W. H. Dietz, *Jama*, 1999, **282**, 1523–1529.
4 D. Mozaffarian, *Am. J. Clin. Nutr.*, 2022, **115**, 1445–1450.
5 L. H. Epstein, C. C. Gordy, H. A. Raynor, M. Beddome, C. K. Kilanowski and R. Paluch, *Obes. Res.*, 2001, **9**, 171–178.
6 H. Boeing, A. Bechthold, A. Bub, S. Ellinger, D. Haller, A. Kroke, E. Leschik-Bonnet, M. J. Müller, H. Oberritter and M. Schulze, *Eur. J. Nutr.*, 2012, **51**, 637–663.
7 R. K. Singh, H.-W. Chang, D. I. Yan, K. M. Lee, D. Ucmak, K. Wong, M. Abrouk, B. Farahnik, M. Nakamura and T. H. Zhu, *J. Transl. Med.*, 2017, **15**, 1–17.
8 C. L. Gentile and T. L. Weir, *Science (80-.).*, 2018, **362**, 776–780.
9 P. J. Turnbaugh, R. E. Ley, M. A. Mahowald, V. Magrini, E. R. Mardis and J. I. Gordon, *Nature*, 2006, **444**, 1027.
10 M. Million, M. Maraninchi, M. Henry, F. Armougom, H. Richet, P. Carrieri, R. Valero, D. Raccah, B. Vialettes and D. Raoult, *Int. J. Obes.*, 2012, **36**, 817.
11 Y. Wu, J. Wan, U. Choe, Q. Pham, N. W. Schoene, Q. He, B. Li, L. Yu and T. T. Y. Wang, *Annu. Rev. Food Sci. Technol.*, 2019, **10**, 389–408.
12 M. C. Kyriacou, Y. Rouphael, F. Di Gioia, A. Kyratzis, F. Serio, M. Renna, S. De Pascale and P. Santamaria, *Trends food Sci. Technol.*, 2016, **57**, 103–115.
13 Z. Xiao, G. E. Lester, Y. Luo and Q. Wang, *J. Agric. Food Chem.*, 2012, **60**, 7644–7651.
14 X. Li, S. Tian, Y. Wang, J. Liu, J. Wang and Y. Lu, *Eur. J. Nutr.*, 2021, **60**, 3829–3839.
15 S. Ma, S. Tian, J. Sun, X. Pang, Q. Hu, X. Li and Y. Lu, *J. Food Biochem.*, 2022, **46**, e14145.
16 S. Verlinden, *Hortic. Rev. (Am. Soc. Hortic. Sci).*, 2020, **47**, 85–124.
17 U. Choe, L. L. Yu and T. T. Y. Wang, *J. Agric. Food Chem.*, 2018, **66**, 11519–11530.
18 E. R. Turner, Y. Luo and R. L. Buchanan, *J. Food Sci.*, 2020, **85**, 870–882.
19 Z. Xiao, S. R. Rausch, Y. Luo, J. Sun, L. Yu, Q. Wang, P. Chen, L. Yu and J. R. Stommel, *Lwt*, 2019, **101**, 731–737.
20 Z. Xiao, E. E. Codling, Y. Luo, X. Nou, G. E. Lester and Q. Wang, *J. Food Compos. Anal.*, 2016, **49**, 87–93.
21 S. A. Mir, M. A. Shah and M. M. Mir, *Crit. Rev. Food Sci. Nutr.*, 2017, **57**, 2730–2736.
22 T. N. Le, C.-H. Chiu and P.-C. Hsieh, *Plants*, 2020, **9**, 946.
23 L. A. David, C. F. Maurice, R. N. Carmody, D. B. Gootenberg, J. E. Button, B. E. Wolfe, A. V. Ling, A. S. Devlin, Y. Varma, M. A. Fischbach, S. B. Biddinger, R. J. Dutton and P. J. Turnbaugh, *Nature*, 2014, **505**, 559–563.
24 E. R. Leeming, A. J. Johnson, T. D. Spector and C. I. Le Roy, *Nutrients*, 2019, **11**, 2862.
25 L. Tian, Y. Tan, G. Chen, G. Wang, J. Sun, S. Ou, W. Chen and W. Bai, *Crit. Rev. Food Sci. Nutr.*, 2019, **59**, 982–991.
26 Y. Li, F. Lu, X. Wang, X. Hu, X. Liao and Y. Zhang, *Food Res. Int.*, 2021, **139**, 109941.
27 J. C. Espín, A. González-Sarrías and F. A. Tomás-Barberán, *Biochem. Pharmacol.*, 2017, **139**, 82–93.
28 I. Epriliati and I. R. Ginjom, *Phytochem. Glob. Perspect. Their Role Nutr. Heal.*, 2012, 401–428.
29 E. Le Chatelier, T. Nielsen, J. Qin, E. Prifti, F. Hildebrand, G. Falony, M. Almeida, M. Arumugam, J.-M. Batto and S. Kennedy, *Nature*, 2013, **500**, 541.
30 F. Liu, Z. Li, X. Wang, C. Xue, Q. Tang and R. W. Li, *Int. J. Mol. Sci.*, 2019, **20**, 2130.
31 J. F. Garcia-Mazcorro, D. Mills and G. Noratto, *FEMS Microbiol. Ecol.*, 2016, **92**, fiw089.
32 J. Li, B. Pang, D. Shao, C. Jiang, X. Hu and J. Shi, *Int. J. Biol. Macromol.*, 2020, **147**, 1008–1017.
33 Y.-J. Zhang, S. Li, R.-Y. Gan, T. Zhou, D.-P. Xu and H.-B. Li, *Int. J. Mol. Sci.*, 2015, **16**, 7493–7519.
34 J. A. Parnell and R. A. Reimer, *Br. J. Nutr.*, 2012, **107**, 601–613.
35 J.-H. Wang, S. Bose, G.-C. Kim, S.-U. Hong, J.-H. Kim, J. Kim and H. Kim, *PLoS One*, 2014, **9**, e86117.
36 Y. Wu, R. W. Li, H. Huang, A. Fletcher, L. Yu, Q. Pham, L. Yu, Q. He and T. T. Y. Wang, *Nutrients*, 2019, **11**, 467.
37 R. W. Li, S. Wu, W. Li, K. Navarro, R. D. Couch, D. Hill and J. F. Urban, *Infect. Immun.*, 2012, IAI-00141.
38 J. Rivera-Pinto, J. J. Egozcue, V. Pawlowsky-Glahn, R. Paredes, M. Noguera-Julian and M. L. Calle, *MSystems*, 2018, **3**, e00053-18.
39 J. Zhou, Y. Deng, F. Luo, Z. He and Y. Yang, *MBio*, 2011, **2**, e00122-11.