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Gut Microbiota Community and Its Assembly Associated with Age and Diet in Chinese Centenarians S

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Increasing evidence suggests that gut microbiota underpin the development of health and longevity. However, our understanding of what influences the composition of this community of the longevous has not been adequately described. Therefore, illumina sequencing analysis was performed on the gut microbiota of centenarians (aged 100-108 years; RC) and younger elderlies (aged 85-99 years; RE) living in Bama County, Guangxi, China and the elderlies (aged 80-92 years; CE) living in Nanning City, Guangxi, China. In addition, their diet was monitored using a semiquantitative dietary questionary (FFQ 23). The results revealed the abundance of *Roseburia* and *Escherichia* was significantly greater, whereas that of *Lactobacillus*, *Faecalibacterium*, *Parabacteroides*, *Butyricimonas*, *Coprococcus*, *Megamonas*, *Mitsuokella*, *Sutterella*, and *Akkermansia* was significantly less in centenarians at the genus level. Both clustering analysis and UniFraq distance analysis showed structural segregation with age and diet among the three populations. Using partial least square discriminate analysis and redundancy analysis, we identified 33 and 34 operational taxonomic units (OTUs) as key OTUs that were significantly associated with age and diet, respectively. Age-related OTUs were characterized as *Ruminococcaceae*, *Clostridiaceae*, and *Lachnospiraceae*, and the former two were increased in the centenarians; diet-related OTUs were classified as *Bacteroidales*, *Lachnospiraceae*, and *Ruminococcaceae*. The former two were deceased, whereas the later one was increased, in the high-fiber diet. The age and high-fiber diet were concomitant with changes in the gut microbiota of centenarians, suggesting that age and high-fiber diet can establish a new structurally balanced architecture of gut microbiota that may benefit the health of centenarians.

Keywords: Centenarians, gut microbiota, illumina sequencing, age, diet

Introduction

Bama Yao Autonomous County, Guangxi, China is the fifth longevous village in the world, as identified at the Thirteenth International Natural Medicine Meeting [1]. According to the Population Census of China in 2010 [34], centenarians accounted for 36.5 out of 1×10^5 people and the longevous phenomenon in the Bama County is more obvious compared with previous reports [40, 41]. Although longevity is associated with many factors, including heredity, diet, society, and the environment [10], the gut microbiota is considered a possible determining factor [4, 14, 40, 41].

Almost inconceivable numbers of microorganisms (10 times greater than the total human eukaryotic cells) inhabit the human intestine [2], where they anaerobically digest food ingredients undigested in the upper gastrointestinal tract, resulting in improved nutrient absorption [36], accelerated physiological chemical transformation, enhanced

host self-repair, and regulated immunity [2].

Colonization of the gut microbiota is considered to be deeply rooted in the evolution of human beings [20]. Recent studies on the gut microbiota in human beings have found that their population is associated with numerous factors, including age [40, 41], region [40, 41], disease [21], and antibiotic use [12]. Researchers have made efforts to search for gut microbiota structure possibly related with age and region by comparing their abundance among centenarians, younger elderly, and adults, based on culture- or PCR-based techniques, even though they only provided incomplete information on microbial diversity [14, 40, 41]. Furthermore, age-related differences were also observed in centenarians living in Italy, using the Human Intestinal Tract Chip (HITChip) and quantitative PCR assays [4], revealing high amounts of opportunistic pathogens and a relocation of *Firmicutes* population. These findings suggest that the two main factors affecting the microbiota structure of healthy subjects are age and region, where the latter includes lifestyle and dietary factors. However, only a few studies have explored the association of the gut microbiota in centenarians with age, and even fewer have investigated the correlation of the composition of gut microbiota with diet.

In the current study, illumina sequencing was used to investigate the differences in gut microbiota among centenarians, Bama younger elderly, and Nanning elderly and to pinpoint the association between different ages and diets and a specific combination of populations in the gut microbiota. Our findings may help to identify specific microbiota signatures for Chinese centenarians and their relationships between diet and longevity.

Materials and Methods

Sampling

A total of 24 subjects were classified according to their age and region as follows: Group RC (n = 8), aged 100–108 years, from the Jiazhuan village of Bama Yao Autonomous County, Guangxi, China, a Bama suburb; Group RE (n = 8), aged 85–99 years, living in the Jiazhuan and Xishan village of Bama; and Group CE (n = 8), the urban elderly, aged 80–92 years, living in Xixiangtang and Langdong district of Nanning City, Guangxi, China (Table S1). The healthy elderly populations were selected from the same socially homogeneous growth environment and eating diet. A detailed clinical examination and medical history were obtained from these elderly's family members, which showed all participants were healthy. None of participants had had gastrointestinal tract disorders, hypertension, diabetes, or other systemic diseases. All participants provided written informed consent prior to study entering and had not taken antibiotics or probiotics one month before the sampling dates. The study was approved by the Ethics Committee of Bama Yao Autonomous County government of Guangxi Province, China.

Fecal samples were collected in sterile containers with ice box by the subjects themselves or their family members, and immediately stored at −20°C. The samples were then transported to the laboratory by a team member within 1–2 h of sampling and preserved at −80°C until extraction of genomic DNA.

The habitual long-term dietary information was obtained from all of the participants or their family members using a semiquantitative dietary questionary (FFQ 23) (Table S2).

DNA Extraction

The genomic DNA was extracted according to the manufacturer's instructions of the Stool DNA Kit (TIANamp Beijing, China) with slight modification as follows: (i) bacteria cells were split at 95°C for 10 min; (ii) additional 4 µl of RNase A was added in the stool sample; and (iii) samples were incubated at 70°C for 30 min. DNA integrity and size were verified by 0.5% agarose gel electrophoresis and DNA concentrations were determined using NanoDrop spectrophotometry (NanoDrop Technologies, USA). The DNA was stored at -20°C.

PCR Amplification of V4 Region of Bacterial 16S rRNA Gene and Illumina Sequencing

The bacteria genomic DNA was amplified with the 515F and 806R primers specific for the V4 hypervariable regions of the 16S rDNA gene. In order to distinguish each sample and yield accurate phylogenetic and taxonomic information, the reverse primer contained a 6 bp error-correcting barcode unique to each sample. All DNAs were amplified by following a protocol described previously [6]. Twenty-four samples were sequenced on an Illumina MiSeq platform according to the manufacturer's recommendations, provided by Beijing Novogene Genomics Technology Co. Ltd (China).

Bioinformatic Analysis

Pairs of reads from the original DNA fragments were merged by using FLASH [25], which was designed to merge pairs of reads when original DNA fragments were shorter than twice the read length. Sequencing reads were assigned to each sample according to the unique barcode and were analyzed with the QIIME [5] software package (Quantitative Insights Into Microbial Ecology) and UPARSE pipeline [17]. In brief, the reads were filtered by QIIME quality filters using default setting for Illumina processing, and operational taxonomic units (OTUs) were picked using the UPARSE pipeline. A total of 802,799 ± 100,349, 539,472 ± 67,434, and 393,005 ± 49,125 sequences from the stool samples of centenarians, Bama younger elderly, and Nanning elderly, respectively, were selected, and the sequences trimmed to 253 bp and assigned to OTUs at 97% similarity (Table 2). A representative sequence was picked for each OTU and assigned to taxonomic

Table 1. The average percentage of protein, fat, carbohydrate, and fiber in centenarians, Bama younger elderly, and Nanning elderly.

	Centenarians (RC)	Bama younger elderly (RE)	Nanning elderly (CE)
Protein %	6.8 ± 1.7%	6.6 ± 1.8%	7.1 ± 1.8%
Fat%[a]	5.5 ± 1.4%	4.5 ± 0.81%	3.9 ± 0.68%
Carbohydrate%[b,c]	13.5 ± 1.6%	17.3 ± 3.8%	12.6 ± 1.6%
Fiber%[a,b]	2.3 ± 0.18%	2.3 ± 0.23%	1.8 ± 0.20%

[a]Indicates statistical significance for both group RC and group CE.

[b]Indicates statistical significance for both group RE and group CE.

[c]Indicates statistical significance for both group RC and group RE.

data using the RDP classifier [37]. The diversity and composition of the bacterial communities were determined using the protocol described by Caporaso *et al*. [6]. In addition, both weighted and unweighted UniFrac were calculated with QIIME to cluster samples using principal coordinate analysis (PCoA) and unweighted pair group method with arithmetic mean (UPGMA).

Multivariate Statistical Analysis

OTUs were identified using partial least square discriminate analysis (PLS-DA) using Simca-p+11.5. Variable importance in projection (VIP) reflects the variables with the most significant contribution in discriminating. Those OTUs with the value of VIP higher than 1.5 were considered as key OTUs. Correlations between key OTUs and age or diet were examined based on t-value analysis obtained from redundancy analysis (RDA) and preformed using Canoco 4.5 (Biometrics, The Netherlands). Using these key OTUs, a heatmap was constructed with HemI 1.0.

Statistical analyses were performed with SPSS 19.0 for Windows (SPSS Inc., USA) to determine the statistical differences among the three groups using parametric methods (ANOVA) and nonparametric statistical methods (Mann–Whitney U-test and Kruskal–Wallis test).

Results and Discussion

Characterization of Dietary Habits

To characterize the diet habits of the 24 elders, a survey with food frequency questionnaire (FFQ23) (Tables S2 and S3) was performed. The average percentage of fiber in the Bama diet was 2.3% in RC and 2.3% in RE, significantly higher than that of 1.8% ($p < 0.05$) in CE. The average percentage of fat in the diet was 5.5% in RC, significantly higher than that of 3.9% ($p < 0.05$) in CE, and the average percentage of carbohydrate in RE was significantly higher than that of 13.5% ($p < 0.05$) and 12.6% ($p < 0.05$) in RC and CE, respectively (Table 1). Although the average percentage of fat and carbohydrate were different between centenarians and Bama young elderly, compared with that in the urbanized world, the fiber average content was similar among the elderly living in Bama village. In addition, their diets were rich in legumes, cereals, polenta, red meat, and vegetables (dark and light vegetable), all of which were completely self-produced, cultivated and harvested, and considered as high-fiber diet. Separately, the diets of Nanning elderly mainly included dairy products, poultry, egg, fish, cereals, dark vegetables, and fruits, which were purchased from a large local farmers' market and was considered as urbanized diet.

Statistical Characteristics of Illumina Sequencing Data

We observed that the gut microbiota of centenarians was more diverse than that of younger elderly. The observed species matrix, which was simplified as the count of the unique OTU numbers, showed an obvious increase in RC and the Chao 1 index was significantly higher in RC and

Table 2. Illumina sequencing data summary.

	Centenarians (RC)	Bama younger elderly (RE)	Nanning elderly (CE)
Sequences produced	100,349 ± 48,106	67,434 ± 8,967	49,125 ± 20,795
OTU number[a]	479 ± 55	460 ± 38	408 ± 34
Chao 1[a,b]	560 ± 77	539 ± 65	451 ± 51
Shannon index (H)	5.45 ± 0.71	5.59 ± 0.63	5.78 ± 0.78

The OTU number, richness estimates (Chao 1), and diversity estimates (Shannon index) were calculated at 3% distance.

[a]Indicates statistical significance for both group RC and group CE.

[b]Indicates statistical significance for both group RE and group CE.

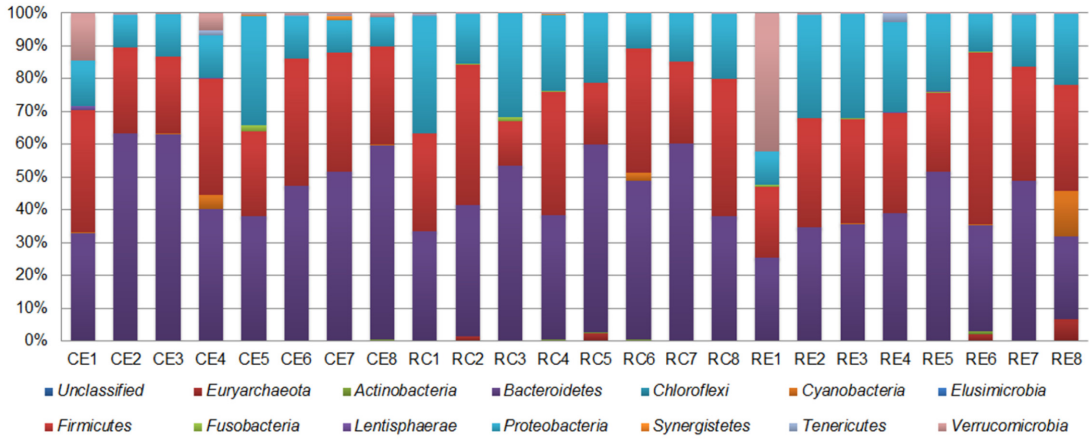

Fig. 1. Relative abundance of the most abundant bacterial phyla in RC1–RC8 (centenarians), RE1–RE8 (Bama younger elderly), and CE1–CE8 (Nanning elderly).

RE ($p < 0.01$), although the species diversity in a community, as estimated by the Shannon index, was similar among RC, RE, and CE (Table 2). For most samples, no curves plateaued with the sequencing (Figs. S1 and S2), while the Shannon diversity started to level off, indicating that although new phylotypes could be found in additional sequencing, most diversity had been well captured with the sequencing effort (Fig. S3). Gut microbial community is a mini-ecosystem whose diversity is regarded as a key health indicator of healthy individuals and affected by the health status of the hosts [35]. It has been reported that reduced biodiversity of the gut microbiota is associated with disease status, including obesity and antibiotic treatment [12, 35]. In contrast, previous studies have suggested that the microbial diversity of gut microbiota was significantly reduced [3, 4, 26] in centenarians and younger elderly although they are healthy.

Distinctive Fecal Microbial Communities Associated with Centenarians

Taxonomically, 14 bacterial phyla were observed in all samples by using Illumina sequencing of the 16S rRNA V4 region (Fig. 1), and more than 94.89% of the sequences in all of the centenarians and younger elderly were found belonging to the three most populated bacterial phyla, namely *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*, in agreement with previous studies showing that such phyla accounted for the majority of gut microbiota in the elderly [4, 29]. *Bacteroidetes* was the most predominant phylum in all samples, followed by *Firmicutes* and *Proteobacteria*. The relative abundance of the *Bacteroidetes* was greater in CE than in RE ($p < 0.05$), whereas no significant difference was found in other dominant phyla among the three groups.

The relative abundance of *Enterobacteriaceae*, *S24-7*, *Methanobacteriaceae*, and *Comamonadaceae* was found to be significantly greater in group RC than group CE, whereas the other seven families *Verrucomicrobiaceae*, *Veillonellaceae*, *Rikenellaceae*, *Porphyromonadaceae*, *Barnesiellaceae*, *Odoribacteraceae*, and *Alcaligenaceae* were significantly lower in group RC than group CE ($p < 0.05$; Table 3). In addition, whereas the relative abundance of *Verrucomicrobiaceae*, *Rikenellaceae*, *Lactobacillaceae*, and *Barnesiellaceae* were significantly greater in group RE than in group RC, no significant difference was found in other dominant families between the two groups ($p < 0.05$).

The relative abundance of two genera, *Roseburia* and *Escherichia*, was found to be significantly higher in RC than CE ($p < 0.05$; Table 4), whereas the other seven genera *Parabacteroides*, *Butyricimonas*, *Coprococcus*, *Megamonas*, *Mitsuokella*, *Sutterella*, and *Akkermansia* were significantly lower in RC than CE ($p < 0.05$), and *Lactobacillus*, *Coprococcus*, *Faecalibacterium*, *Mitsuokella*, and *Akkermansia* were significantly lower in RC than RE ($p < 0.05$). Moreover, *Methanobrevibacter*, *Lactobacillus*, *Escherichia*, and *Akkermansia* were significantly higher in RE than in CE ($p < 0.05$), whereas *Butyricimonas*, *Lachnospira*, *Megamonas*, *Mitsuokella*, and *Sutterella* were significantly lower in RE than in CE ($p < 0.05$)

We observed that another important characteristic of gut microbiota in centenarians is the structural change in butyrate-producing bacteria in the phylum *Firmicutes*. Among of them, *Clostridium* cluster IV (represented by *Faecalibacterium*) and *Clostridium* cluster XIVa (represented by *Roseburia*) are considered beneficial for energy metabolism

Table 3. List of families that were significantly different among RC, RE, and CE.

Bacterial family	Relative contribution (%)			Median, range (%)		
	RC	RE	CE	RC	RE	CE
Verrucomicrobiaceae[a,b,c]	0.02	5.40	2.91	0.01, 0.00-0.15	0.13, 0.09-42.1	0.68, 0.31-14.37
Enterobacteriaceae[a,b]	18.59	18.74	10.43	16.25, 8.90-32.57	17.15, 8.63-29.73	7.59, 5.97-29.05
Veillonellaceae[a]	2.36	3.34	4.95	1.05, 0.24-10.34	2.46, 1.71-8.55	4.02, 2.20-11.37
S24-7[a,b]	2.86	2.51	1.61	1.08, 0.81-7.53	1.63, 0.56-6.57	0.36, 0.25-9.94
Methanobacteriaceae[a,b]	0.49	1.06	0.00	0.02, 0.00-2.40	0.03, 0.00-6.47	0.00, 0.00-0.03
Rikenellaceae[a,c]	0.73	3.07	3.04	0.59, 0.21-1.61	3.06, 0.59-5.60	3.09, 0.55-6.18
Lactobacillaceae[b,c]	0.00	0.95	0.04	0.00, 0.00-0.01	0.27, 0.00-6.02	0.01, 0.00-0.27
Comamonadaceae[a,b]	0.70	1.41	0.07	0.21, 0.15-4.13	0.94, 0.00-5.74	0.00, 0.00-0.50
Porphyromonadaceae[a]	0.58	0.73	2.13	0.55, 0.21-1.02	0.58, 0.39-1.68	1.30, 0.40-5.42
Barnesiellaceae[a,c]	0.27	0.53	1.33	0.11, 0.06-1.17	0.48, 0.18-1.00	0.77, 0.48-3.82
Odoribacteraceae[a,b]	0.18	0.40	1.36	0.15, 0.05-0.52	0.31, 0.11-1.00	1.00, 0.17-3.30
Catabacteriaceae[b]	0.63	0.27	0.85	0.31, 0.16-2.97	0.21, 0.14-0.79	0.39, 0.16-2.27
Alcaligenaceae[a,b]	0.21	0.31	1.89	0.18, 0.06-0.42	0.25, 0.10-0.89	1.96, 0.92-2.76

Abbreviations: RC, Centenarians; RE, Bama younger elderly; CE, Nanning elderly.

[a]Indicates statistical significance for both group RC and group CE.

[b]Indicates statistical significance for both group RE and group CE.

[c]Indicates statistical significance for both group RC and group RE.

Table 4. List of genera that were significantly different among RC, RE, and CE.

Bacterial genera	Relative contribution (%)			Median, range (%)		
	RC	RE	CE	RC	RE	CE
Methanobrevibacter[b]	0.49	1.06	0.01	0.02, 0.00-2.40	0.03, 0.00-6.48	0.00, 0.00-0.03
Parabacteroides[a]	0.58	0.73	2.12	0.56, 0.21-1.02	0.59, 0.39-1.68	1.27, 0.40-5.40
Butyricimonas[a,b]	0.12	0.31	1.28	0.11, 0.05-0.24	0.19, 0.07-0.89	0.89, 0.19-3.15
Lactobacillus[b,c]	0.00	0.95	0.04	0.00, 0.00-0.01	0.27, 0.00-6.01	0.01, 0.00-0.27
Coprococcus[a,c]	0.29	1.25	1.14	0.26, 0.10-0.68	1.20, 0.60-2.03	1.10, 0.47-1.77
Lachnospira[b]	0.89	0.44	1.17	0.75, 0.33-1.79	0.40, 0.20-1.06	1.00, 0.61-2.64
Roseburia[a]	3.55	2.77	1.54	3.34, 1.14-6.18	2.39, 0.95-5.11	1.60, 0.64-2.66
Faecalibacterium[c]	2.98	5.24	5.25	1.89, 0.81-8.60	3.97, 2.36-13.56	4.69, 1.43-10.48
Megamonas[a,b]	0.00	0.02	1.24	0.00, 0.00-0.00	0.00, 0.00-0.14	0.29, 0.00-7.88
Mitsuokella[a,b,c]	0.00	1.13	0.21	0.00, 0.00-0.00	0.45, 0.10-5.89	0.10, 0.06-0.73
Sutterella[a,b]	0.21	0.32	1.89	0.19, 0.06-0.44	0.25, 0.10-0.88	1.96, 0.91-2.77
Escherichia[a,b]	13.78	13.86	6.78	10.55, 5.14-27.03	11.17, 5.93-25.32	4.64, 2.78-24.35
Akkermansia[a,b,c]	0.02	5.39	2.91	0.01, 0.00-0.15	0.13, 0.09-42.09	0.68, 0.30-14.36

Abbreviations: RC, Centenarians; RE, Bama younger elderly; CE, Nanning elderly.

[a]Indicates statistical significance for both group RC and group CE.

[b]Indicates statistical significance for both group RE and group CE.

[c]Indicates statistical significance for both group RC and group RE.

and normal development of colonic epithelial cells [28], the reduced amounts of which have been noticed in frail elderly and patients with colorectal cancer and liver cirrhosis [7, 38, 42]. The abundance of genus *Faecalibacterium*

of *Clostridium* cluster IV was less in centenarians than in young elderly, which is in accordance with a previous research [4]. In contrast, compared with younger elderly, we observed that the abundance of *Roseburia* of *Clostridium* cluster XIVa was greater in centenarians ($p < 0.05$). Many researchers have found the importance of *Roseburia* in the production of butyrate and conjugated linoleic acids [13, 15]. A previous study also found that the relative high abundance of *Clostridium coccoides* and *Eubacterium rectale* of *Clostridium* cluster XIVa is clearly greater in centenarians than Bama younger elderly based on the analysis using culture technology [40]. However, a significant reduction of bacteria of *Clostridium* cluster XIVa in Italian centenarians has been noticed [4]. The discrepancy of *Clostridium* cluster IV and *Clostridium* cluster XIVa in gut microbiota of centenarians from different geographic provenience may be related to different dietary habits.

We also observed that *Bacteroidetes* was more commonly detected in centenarians than in young elderly from the same area, although lower levels of *Rikenellaceae* and *Porphyromonadaceae* were seen in centenarians ($p < 0.05$). *Bacteroidetes* species in the gut microbiota play an important role in carbohydrate fermentation, catabolism of polysaccharides, bile acids, and cholesterol, and utilization of amino acids and proteins, and could affect human health [32]. Some evidence showed that *Bacteroidetes* in elderly individuals tends to be dominated, differing from that of younger individuals [8, 26], and consistent with our comparison results. However, an age-related reduction in *Bacteroidetes* was found in Italian elderly and centenarians [4, 26]. In addition, consistent with the previous results [4, 14], *Proteobacteria* was more commonly detected in centenarians, such as *Enterobacteriaceae* was more abundant in centenarians who lived in the ancient communities that usually had poor hygiene environments and thus could favor the spread of these species.

The family *Methanobacteriaceae* was not present in the Nanning elderly, but its level was more abundant in centenarians. The influence of diet on the gut microbiota has been found in previous studies. For example, fermentation of complex polysaccharides results in the production of primarily acetate, propionate, and butyrate, normally in a ratio of 3:1:1 [33]. The high-fiber diet may support *Methanobacteria* strains, which utilize acetic acids and produce methane in centenarians and in the environment of ancient communities where intestinal archaea can be dominant. Conversely, the relative abundance of *Megamonas* and *Mitsuokell* within the family *Veillonellaceae* was obviously lower in centenarians ($p < 0.05$). The enrichment of family *Veillonellaceae* in gut micobiota, typical small intestine bacteria [24], may be bowel movements at a slower pace [7]. Hence, the decrease of *Veillonellaceae* in centenarians suggested their bowel movements at a regular pace. Our results also showed that the level of *Akkermansia*, a mucin-degrading bacterium, was relatively low in centenarians compared with that in younger elderly ($p < 0.05$), which was in line with a previous study [9], but contrary to another study [4], showing that the level of *A. muciniphila* was more abundant in aged people. Hence, whether the decrease of *Akkermansia* in centenarians is correlated to host aging and longevity would require further investigation.

Structural Comparison of Gut Microbiota among RC, RE, and CE with Multivariate Statistics

Differences in the total bacterial community at the single sample level were assessed by a net separation of the three groups produced by the RC, RE, and CE samples clustering and a complete linkage hierarchical clustering according to their bacterial genera as found by the RDP classifier (Fig. 2A). It was noteworthy that the samples could be grouped into two distinct clusters. Each cluster contained subjects of the same dietary habit (ignoring CE1 and RE1), indicating the effect of dietary habit on the gut microbiota community. It was also noteworthy that one of the clusters was separated, because the RE elderly and RC elderly belonged to different age groups. The results indicated a dominant role of age in shaping the composition of gut microbiota. Similar results were also obtained in a PCoA-based UniFraq distance analysis (Fig. 2B).

Supporting these observations, associations between the structural shift of gut microbiota with different age and diet in human beings, including infants [18, 19, 27], elderly [8], and residents with different diets [11, 30], have been highlighted by several earlier studies. In addition, alterations of gut microbiota also associated with age have been suggested in centenarians in comparison with other adults and younger elderly using culture-dependent [14, 41] and -independent molecular approaches [4, 40].

Correlations of Relative Abundance of Bacterial Groups with Age and Diet

PLS-DA, a multivariate analysis method, was used to identify the key OTUs responsible for differences in the gut microbiota. A total of 54 key OTUs with a VIP > 1.5 were identified and used to identify the specific bacteria with intrinsic difference in gut microbiota compositions among

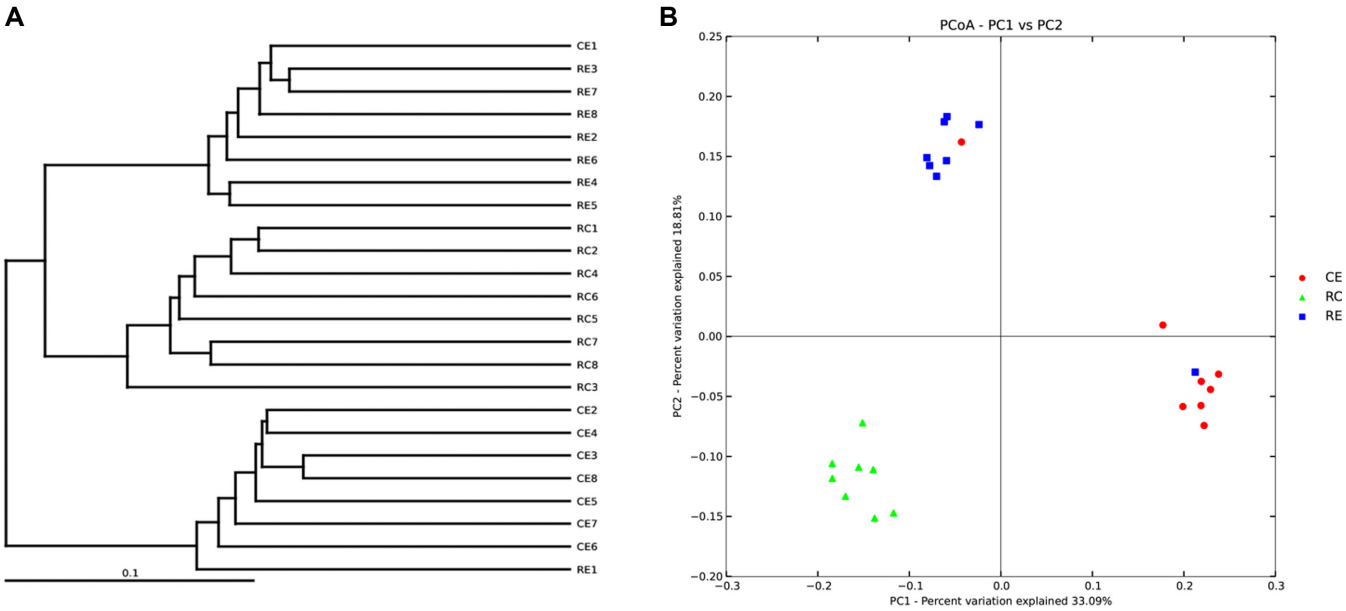

Fig. 2. 16S rRNA gene surveys reveal a clear separation of the three elderly populations investigated.
(**A**) Dendrogram obtained with complete linkage hierarchical clustering of the samples from centenarians (RC1–RC8), Bama younger elderly (RE1–RE8), and Nanning elderly (CE1–CE8) based on the total OTUs. (**B**) Principal coordinate analysis (PCoA) plot based on the weighted UniFrac metric.

groups with different age and diet. Redundancy analysis using the key OTUs as the species variable and age and region as the environmental variables showed that both the constrained ordination models by age and diet were significant ($p = 0.002$ and $p = 0.002$).

The RDA indicated that 33 OTUs of gut microbiota in the three groups were significantly correlated with age (Fig. 3A; $p < 0.05$). Nineteen OTUs were more abundant and 14 OTUs were less in RC. Most of these OTUs were classified to the families *Ruminococcaceae* (12 OTUs), *Lachnospiraceae* (6 OTUs), and *Clostridiaceae* (4 OTUs). OTUs from family *Ruminococcaceae* were identified as the key specific gut

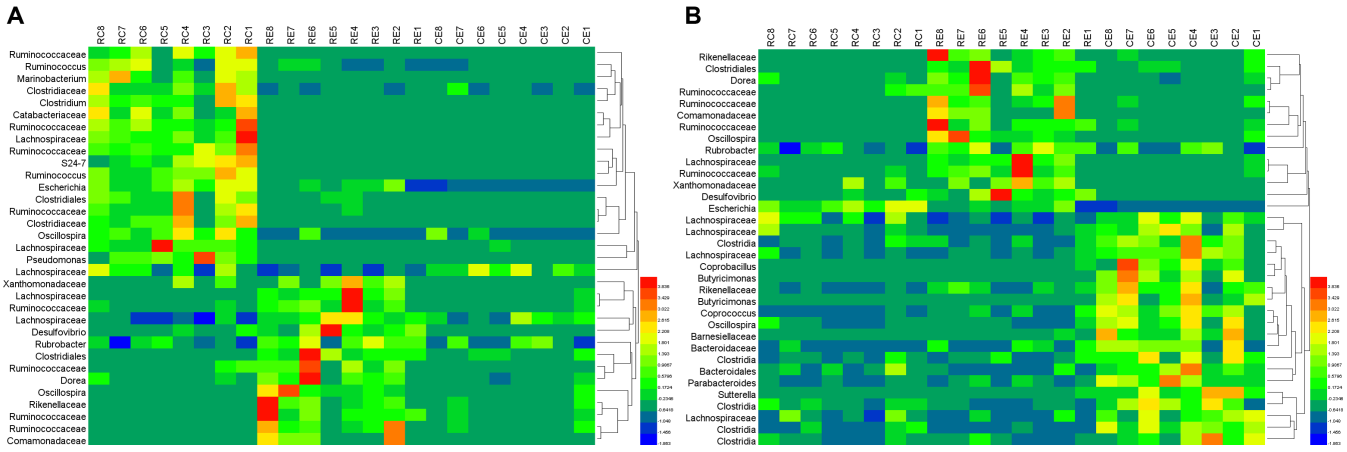

Fig. 3. Heatmap constructed using the key OTUs of elders that correlated with age and diet.
The order, family, and genus names of the key OTUs are shown on the left at the corresponding position. Individuals are shown at the top of the heatmap. (**A**) Relative abundances of the 33 OTUs identified as key variables for the differentiation of microbiota of centenarians (RC1–RC8) and younger elderly (RE1–RE8 and CE1–CE8). (**B**) Relative abundance of the 34 OTUs identified as key variables for the differentiation of microbiota of high-fiber diet (RC1–RC8 and RE1–RE8) and urbanized diet (CE1–CE8).

microbiota, among which seven OTUs were highly abundant and five OTUs less abundant in centenarians, and OTUs from *Clostridiaceae* disappeared in younger elders. In addition, there were 25 OTUs that were significantly different between centenarians and younger elderly (Table S4).

As shown in Fig. 3B, the abundance of 34 OTUs were significantly correlated with the diet of the elderly ($p < 0.05$). The abundance of 14 of these OTUs was greater, and of 20 was less, in the fiber diet group than in urbanized diet group. Most of these OTUs were distributed across order *Bacteroidales* (8 OTUs) and families *Lachnospiraceae* (7 OTUs) and *Ruminococcaceae* (6 OTUs). The abundance of these selected phylotypes in *Bacteroidales* and *Lachnospiraceae* was lower in the high-fiber diet group, but the OTUs of *Ruminococcaceae* were higher in the high-fiber diet group. Twenty-six of these OTUs were significantly different between the high-fiber diet and urbanized diet groups (Table S5).

The family *Ruminococcaceae* of *Clostridium* cluster IV has been known to degrade dietary fiber and produce SCFA [22], and the higher abundance of *Ruminococcaceae* in subjects on diets supplemented with resistant starch had also been described by Salonen *et al*. [31], which was in line with our current study. Additionally, OTUs classified to *Lachnospiraceae* of *Clostridium* cluster XIVa were found to contribute to the structural differences between gut microbiota of different age and diet groups based on the results of Heatmap. The health-promoting functions of *Lachnospiraceae* include participating in carbohydrate fermentation into short-chain fatty acids, CO_2, and H_2, resulting in increasing nutrients for the host and modulating colonic pH [16, 39]. Although having no significant difference among the three groups, OTUs related to *Lachnospiraceae* were less detected in high-fiber diet. Hence, the peculiar compositional layout of butyrate-producing bacteria associated with age and diet in centenarians may contribute to modulating the structure of the gut microbiota to a more balanced state.

OTUs closely related to *Clostridiaceae* were more abundant in the gut microbiota of centenarians. Although the prevalence of *Clostridiaceae* is positively associated with infancy food allergy, there is still no sufficient evidence about the role of *Clostridiaceae* in pathogenicity [21]. In addition, in the case of high-fiber diet, *Bacteroidetes* was found to be less, although it has always been regarded as the most abundant in polysaccharides-rich diet [11]. Bacteria within *Bacteroidetes* were adept in degrading resistant starch in the human large intestine [23] and dominant species vary in the ability to utilize different types of starch substrates [33]. Therefore, we speculated that the decreased OTUs of *Bacteroidetes* in high-fiber diet group might originate from different types of dietary carbohydrates.

Our results point to a new balanced gut microbiota architecture of Chinese centenarian induced by different ages and diets. Given the potential key role of a high-fiber diet in mediating longevity, it will be of interest to the general public and become a possible guide of dietary interventions for health and longevity in the future.

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