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Bacterial and Archaeal Diversities in Maotai Section of the Chishui River, China

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Abstract The Chishui River is the last undammed tributary of the upper Yangtze River, extends cross Sichuan, Yunnan and Guizhou provinces, and it is the significant water source of Maotai liquor, Southwest of China. We investigated microbial community of the Chishui River in the Maotai town section, because of deep relationship between the water and the most famous Chinese liquor, Maotai liquor. In this study, diversities of bacteria and archaea of four seasons were analyzed in two different sampling sites using a barcoded 16S rRNA gene-pyrosequencing approach. The results show that the predominant community among all bacteria is *Proteobacteria* (70.16–94.29 %), of which *Gamma-proteobacteria* made up the largest portion. Bacterial community structure in spring and autumn tended to group together, and the operational taxonomic units of bacteria peaked in summer. The quantitative PCR (q-PCR) results revealed significantly higher number of gene copies in the downstream than that in the upstream, and were slightly higher in summer and spring than other seasons. Archaeal community structures had no obvious regular pattern, and species richness was higher in downstream in all seasons. *Euryarchaeota* and *Thaumarchaeota* were the dominant groups in archaeal populations, and abundant ammonia-oxidizing archaea was detected. The study significantly improved our understanding of microbial community in Maotai section of the Chishui River, where the unique and world-famous Maotai liquor is produced.

Introduction

The Chishui River (27°20′–28°50′ N; 104°45′–106°51′ E) is the first level tributaries of the Yangtze River, originates in north of the Wumeng Mountains of Yunnan Province, Southwest of China. The river is divided into three reaches: the upstream reach is from the headwaters to the Maotai town, the middle reach is from Maotai town to Chishui city, the downstream segment extends from Chishui to Hejiang [34]. The Chishui River is famous as the "Eco-river with a lot of endemic fishes", "Beauty River with natural scenic landscapes" and "Chinese famous liquors river with Maotai" [25]. In 1974, Chinese premier Zhou Enlai commanded to develop the production of Maotai (Moutai) liquor up to ten thousand ton, and attempt to

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move Maotai distillery to Zunyi city, which possessed better economy at that time. However, after an eleven-year experiment, it failed to repeat the unique flavor of Maotai. Maotai liquor is unique owe to natural environmental conditions, such as climate, terrain and source of water—the Chishui River. The microbial community of the Chishui River is a crucial part of Maotai liquor production, which attracts researchers to investigate.

Diversity of microbial communities of some famous freshwater rivers or lakes have been reported, such as the Changjiang River [28], the Mississippi River [29, 30], the Oilo River [27] and the Mooi River [21]. Many molecular methods including denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE) and fluorescent in situ hybridization were applied to investigate the microbial community structure. However, these low-throughput approaches are not able to completely reveal the detailed microbial community structure due to the extremely complex communities and overwhelming genetic diversities, and the approaches based on clone library sequencing of the 16S rRNA gene for ecological investigations of functional microorganisms may result in an overestimation or underestimation of both their numbers and the diversity due to their inherent bias of amplification [16]. With the development of the second generation sequencing technology recently, some studies which employed 454 pyrosequencing technologies to explore microorganisms were reported [2, 16, 27, 29, 30, 32].

Research on the microbial community structure and diversity of the Maotai section of the Chishui River is not only related with Maotai liquor's unique taste and flavor, but also is expected to provide fundamental information for the management and protection of the Chishui River. In this study, fresh water samples collected from the Chishui River at Zhonghua site (A, upstream, water resource of Kweichow Moutai Co.) and Maotai bridge site (B, downstream, water resource of other Maotai-flavor liquor distilleries) were analyzed physicochemically and microbiologically. This is the first report on the microbial community status of the Chishui River in four seasons analyzed by 454 pyrosequencing and q-PCR technique.

Results

Characteristics of Environment

Two sampling sites (A upstream; B downstream) across four seasons were selected for field measurements and sample collections (Fig. S1). Physicochemical property (temperature, pH, oxidation–reduction Potential, dissolved oxygen, COD_{Mn} and microelement contents) of samples was measured. Results of A and B had little difference in the same season (Table S1). The range of variation of pH, oxidation–reduction potential, dissolved oxygen and the value of COD_{Mn} were relatively low. The detected ion composition fell within normal ranges and river water was oligotrophic according to the COD_{Mn} data.

Overview of Bacterial Communities in Maotai Section of the Chishui River

A total of 25,891 bacterial reads were identified, which can be clustered into 3892 operational taxonomic units (OTUs). Based on the Ribosomal Database Project (RDP), all the obtained bacterial sequences fell into 16 phyla (387 genera) (Fig. S2). *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Firmicutes* were found in all eight samples, of which *Proteobacteria* (including *Alpha*-, *Beta*-, *Delta*-, *Epsilon*- and *Gamma-proteobacteria* in all samples) represented the dominant microflora. A large number of *Gamma-proteobacteria* sequences dominated in bacterial distribution of all samples. In genus level, *Acinetobacter*, *Limnohabitans*, *Sphingorhabdus*, *Flavobacterium*, *Pseudomonas*, *Novosphingobium*, *Brevundimonas*, *Sphingomonas*, *Rhodobacter*, *Polynucleobacter*, *Albidiferax*, *Gemmobacter* and *Caulobacter* were found in all samples, of which *Acinetobacter* and *Limnohabitans* were the dominant bacterial genera.

Annual and Spatial Variability in Bacterial Community Structure

Alpha-diversity was assessed according to Chao, Shannon, ACE and Simpson based on data of OTUs (Table S2). Obviously, richness rarefaction curve (Fig. S3) of bacterial samples did not yet reach saturation plateau, which is due to the limited the number of sequencing. However, slope of OTUs-based richness rarefaction curves indicated that OTUs of bacteria was highest in summer. Bacteria rarefaction curves of Shannon index approached the saturation plateau at a sequencing depth of 500 in all samples. The Shannon diversity index at a sequencing depth of 2274 and its rarefaction curves showed that the bacterial diversity of the summer and winter was significantly higher than the indices of other two seasons (Fig. S3b). From the above analysis, it can be concluded that the summer had the highest bacterial community diversity and richness.

Cluster heatmap analysis (Fig. S4) and principal coordinates analysis (PCoA) (Fig. S5) were performed to visualize the differences in the eight bacterial community structures. Seasonal variation of community structures was apparent. The difference between upstream (A) and downstream (B) in summer and winter was greater than that in spring and autumn. Clustering analysis showed samples from site A and site B clustered in all four seasons. Principal coordinates analysis also showed samples from spring and autumn tended to group together. On the other hand, samples from summer and winter had no regular pattern in and PCoA. In summer samples, OTUs (42.27 %) assigned mainly to *Limnohabitans* (7.08 %) and unclassified *Comamonadaceae* (4.49 %) were present only in A, and some OTUs (43.61 %) assigned primarily to *Acinetobacter* (7.50 %) and unclassified *Enterobacteriaceae* (4.66 %) were unique in B. In winter samples, OTUs (27.87 %) assigned mainly to *Acinetobacter* (5.11 %), *Limnohabitans* (6.06 %) and unclassified *Comamonadaceae* (2.30 %) were unique in site A, while exclusive OTUs (52.4 %) of site B were assigned primarily to *Acinetobacter* (17.11 %), unclassified *Sphingomonadaceae* (6.50 %) and *Lactococcus* (3.37 %). There was little difference between A and B in autumn and spring, PCoA analysis was consistent with network analysis (Fig. S5c, d). Mutual OTUs of all samples were assigned mainly *Acinetobacter* and *Limnohabitans*, and the OTUs of *Acinetobacter* and *Limnohabitans* were significantly different in different sites and seasons, which decided the whole bacteria community structure.

16S rRNA Gene Abundance Analyzed by q-PCR

Change of 16S rRNA gene copies of bacteria at different sites in different seasons is shown in Fig. S6. The maximum number of $(5.23 \pm 0.16) \times 10^8$ copies/L appeared in SU-B, and the minimum of $(4.12 \pm 1.53) \times 10^6$ copies/L appeared in AU-A. Bacteria abundance in the four different seasons showed significant differences (P value <0.05), of which samples of downstream had a higher number.

Composition of Archaeal Communities in Maotai Section of the Chishui River

A total of 43,462 reads of archaeal 16S rRNA gene sequence, which belonged to 5586 OTUs (cut-off 97 %) and 36 genera, were identified. The dominant phyla were *Euryarchaeota* and *Thaumarchaeota*, of which dominant genera comprised *Nitrososphaera*, *Methanospirillum*, *Nitrosopumilus*, *Methanobacterium*, *Methanoregula*, *Methanosphaerula* and unclassified *Thermoprotei* (Fig. S7).

Seasonal Spatiotemporal Variation in Archaeal Community Structure

Archaea data of OTUs, and Chao, Shannon, Simpson and ACE results are presented in Table S2. The OTUs of archaea in summer were higher than that of other seasons. The OTUs-based richness rarefaction curve of archaea samples (Fig. S8a) indicated that archaea were more diverse in summer, followed by spring, winter and autumn. In addition, species richness was higher in site B every season, whose value is about 3–4 times as large as that in A. Archaeal rarefaction curves of Shannon index approached the saturation plateau at a sequencing depth of 1000 in all samples. The Shannon diversity index at a sequencing depth of 4761 and its rarefaction curves showed that the archaeal diversity of the summer was significantly higher than the index in other seasons, and archaeal diversity of site B was higher than that in site A (Fig. S8b). It can be inferred that archaeal community diversity and richness of B was higher than that in A. In addition, archaeal diversity of summer was significantly higher than other seasons.

Unlike bacteria, the similarity between the communities in the samples collected in the same season is inconspicuous except in summer according the cluster heatmap analysis (Fig. S9). However, archaea community structures were relatively similar among SU-A, AU-A and SP-A, as well as SU-B, AU-B and WI-B in the PCoA (Fig. S10a, b), which demonstrated seasonal variation was not the primary factor in influencing major archaea communities. Apparently, SU-A and SU-B clustered in the heatmap, and the radio of *Thaumarchaeota* was relatively high in summer. According to statistics, common OTUs of site A across all four seasons were low and belonged to *Nitrososphaera*, and it was more abundant in autumn (3.30 %) and spring (8.71 %) than that in summer (0.56 %) and winter (0.72 %). Common OTUs of B was more diverse, and high number of unique OTUs were observed in A and B in the same season (Fig. S10c, d).

Discussion

The primary environmental characteristics of Maotai section of the Chishui River are the temperature, which varies greatly between spring/summer and autumn/winter. Investigation on the physicochemical property of the environment shows that many properties including metallic element were relatively stable in every sample. However, river water is oligotrophic by COD_{Mn} data and element determination, which implies that the water body was not suitable for the growth of most heterotrophic microorganism.

High-throughput 454 pyrosequencing technology has been recently applied as an advanced method to characterize the phylogenetic composition and functional potential of complex community, and data generated by this technology reveals greater details than conventional clone library and DGGE approaches [2, 16, 32]. Using this technology, we found in this study that bacteria species were abundant,including 3892 OTUs. The result is in consistent with other studies that suggest high levels of bacterial diversity in freshwater environments [9, 20]. Dominance of bacterial groups has been analyzed in freshwater bodies in many studies. *Beta-proteobacteria* accounted for the largest percentage of sequences in the investigation of bacterial communities of the Mississippi River [29]. In the research of lotic organic aggregates ('river snow') in the Elbe River, *Beta-proteobacteria* has been shown to constitute the numerically largest bacterial group, forming up to 54 % of the total cell count during all seasons [3]. The most abundant bacterial phylum is *Proteobacteria* in this study, which consists of five subdivisions with diverse morphology and function. Proportions of *Beta-proteobacteria* and *Gamma-proteobacteria* from the Chishui River are relatively high, of which the abundance of *Beta-proteobacteria* is similar to that found in other freshwater environments. Nevertheless, in previous research, the frequencies of occurrence of *Beta-proteobacteria* were relatively high and a low frequency of occurrence of *Gamma-proteobacteria* was observed in freshwater [24]. In addition, *Gamma-proteobacteria* is present as a minor group in Changjiang River [28], sediments of the Shenzhen River [8] and the Mooi River [21]. In this study, it is found that *Gamma-proteobacteria* constitutes the numerically most abundant bacterial group in the Chishui River with 42.10–75.25 % among all samples, which is different from other previous freshwater studies. *Acinetobacter* and *Limnohabitans* are dominant in terms of the level of genus. *Limnohabitans* is a planktonic bacterium rarely reported from freshwater pond, reservoir and lake [17, 18, 22].

This is the first report on diversity of the microbial community of the Chishui River (Maotai section). Species diversity and OTU value of bacteria were relatively high in summer, which might be affected by increased flow. Summer is the wet season of the Chishui River catchment, when there is a large amount of sediment input.

The 16S rRNA gene copies of bacteria at two sites in different seasons were calculated and found that the copies in summer and spring were slightly higher than that in autumn and winter. This may imply that growth of bacteria was affiliated with the water temperature and low temperature may limit the growth of some bacteria. Water temperature has been repeatedly suggested as a strong factor for bacteria growth. For example, the bacterial community structure of Mississippi River water samples was observed to be similar in the late summer of 2011 and 2012 suggesting that seasonal parameters, specifically temperature, may result in annually reproducible bacterial [29]. Meanwhile, the copies of bacteria and their diversity were higher in site B than that in site A. Maotai residents live near site B, which means that human activities may cause organic matters to be discharged into river, which may be the cause of the higher bacteria number and diversity. However, the $\mathrm{COD}_{\mathrm{Mn},}$ values of site A and B were not significantly different. This may be due to the strict control of industrial and domestic wastewater discharge into the river. Even though human activities caused a slightly increased bacteria number and diversity at site B, it is still within the self-purification range of the river.

Although not shown in this report, the culturable microorganisms of Chishui River (Maotai section) were also investigated using the same samples. Interestingly, the novel species are abundant in the river. A series of novel species or genus were isolated from water samples of current report by standard dilution plating method and proposed as new taxa in our laboratory, such as *Flavobacterium maotaiense* sp. nov. [10], *Flavobacterium procerum* sp. nov. [11], *Flavobacterium buctense* sp. nov. [12], *Arenimonas maotaiensis* sp. nov. [36], *Taibaiella chishuiensis* sp. nov. [33] and *Chishuiella changwenlii* gen. nov., sp. nov. [38].

The studies about archaea groups in water samples are increasing with the rapid development of high-throughput sequencing technology. However, research on archaea diversity from freshwater using 454 pyrosequencing is still rare. In our study, *Euryarchaeota* and *Thaumarchaeota* were dominantarchaeal phyla. *Euryarchaeota* from the Chishui River, including *Methanosarcina*, *Methanospirillum*, *Methanobacterium*, *Methanosphaerula*, *Methanosaeta* and *Methanoregula*, were affiliated with known methanogenic groups. Methanogenic archaea are obligate anaerobe and common in anaerobichabitats such as soils [15], lake sediments [7], permafrost soils [14], hyporheic sediments [4], and stream sediments [5]. The abundant methanogenic archaeal in this study may well be carried into the water samples from river sediment. It was noteworthy that abundant *Thaumarchaeota* was detected, represented by *Nitrososphaera*, *Nitrosopumilus* and unclassified *Thaumarchaeota*. *Nitrososphaera* and *Nitrosopumilus* were reported as typical ammonia-oxidizing archaea (AOA) [23, 31] and considered to play an important role in the global nitrogen cycle, particularly in the ocean layer [13], estuaries/marine water [6, 26], soil/sediment [1, 35] and lakes [19]. The AOA have contributed to the nitrification in acidic soil from Yunnan [37]. "Laterite plateau" is another name of Yunnan-Guizhou plateau, and Yunnan was the origin of the Chishui River. Laterite soil covers both banks of the Chishui River, and is washed into the river with storm water, which turns water "red", which is called "Chi" in Chinese. "Chishui" literally means "red water". It suggested that *Thaumarchaeota*in the Chishui River is from the acidic laterite soil in the upper Chishui River basin.

Diversity and community structure of archaea did not show any correlation with the seasons in this study. The distribution of archaeal population may relate to the sampling sites and community structure of archaea is more similar in downstream during the seasons than that in upstream. Meanwhile, species richness of B was higher in the same season than that of A. Using q-PCR, the 16S rRNA gene of archaea was barely detectable in the sample. This may be due to low abundance of archaea.

In summary, our study shows that *Gamma-proteobacteria* and *Thaumarchaeota* occupy the largest portion of bacteria and archaea, respectively, which is different from previous research in other freshwater bodies. We also proposed several novel bacteria taxa isolated from these water samples, suggesting that the water of the Chishui River may support some unknown microorganisms growth. Furthermore, these results about diversity of bacteria and archaea community in Maotai section of the Chishui River have provided fundamental information for protection of the Chishui River.

Supporting Information

(1) Experimental procedures.
(2) Supplementary Tables.
(3) Supplementary Figures.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that there is no conflict of interest.

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