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Comparative Study of Oral Bacteria and Fungi Microbiota in Tibetan and Chinese Han Living at Different Altitude

Ke Dong,[1,2] Kunpeng Wu,[1,2] Tianli Zheng,[1,2] Ji Yue,[1,2] Weipeng Wang,[1,2] Ruocheng Luo,[1,2] Lan You,[1,2] Xun He,[1,2] Jingjing Li,[1,2] Zehui Hong,[1,2] Haojiang Zuo[1,2] and Xiaofang Pei[1,2]

[1]West China School of Public Health and West China Fourth Hospital, Sichuan University, Chengdu, Sichuan, China
[2]Food Safety Monitoring and Risk Assessment Key Laboratory of Sichuan Province, Chengdu, Sichuan, China

Knowledge about the impact of altitude and ethnicity on human oral microbiota is currently limited. To obtain the baseline of normal salivary microbiota, we analyzed the bacteria and fungi composition in Tibetan (HY group) and Han population (CD group) living at different altitudes by using next-generation sequencing (NGS) technology combined with PICRUSt and FUNGuild analyses. There were significant differences in oral microbiota composition between the two groups at phylum and genus levels. At the phylum level, the HY group had higher relative abundances of *Firmicutes* and *Ascomycota*, whereas the *Bacteroidetes* and *Basidiomycota* in the CD group were richer. These changes at the phylum level reflected different dominant genus compositions. Compared with the Han population, *Candida*, *Fusarium*, *Zopfiella*, *Streptococcus*, *Veillonella* and *Rothia* in Tibetan were higher. Surprisingly, the *Zopfiella* was found almost exclusively in the Tibetan. The PICRUSt and FUNGuild analysis also indicated that the function of the bacterial and fungal communities was altered between the two groups. In conclusion, our results suggest that there are significant differences in oral microbial structure and metabolic characteristics and trophic modes among Tibetan and Han population living at different altitudes. We first established the oral microbiota framework and represented a critical step for determining the diversity of oral microbiota in the Tibetan and Han population.

Keywords: Internal Transcribed Spacer 1 (ITS1); oral microbiota; plateau environment; saliva; 16S rRNA gene sequencing
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Introduction

The Tibetan Plateau is considered one of the places on earth with the most extreme environmental conditions, which is characterized by low air pressure, low oxygen, and high radiation (Leon-Velarde et al. 2005; Cuo and Zhang 2017). These conditions are formidable physical and mental challenges for residents or newcomers living in this high-altitude plateau (Xu et al. 2015). The plateau's hypobaric hypoxic environments result in decreased arterial oxyhemoglobin saturation, increased heart rate, and Han individuals' hormone disorders, while local Tibetan are barely affected (Huerta-Sanchez et al. 2014). These Tibetan serve as good examples of successful high-altitude adaptation because of their different genotypes formed by long time nature selection (Wu and Kayser 2006). Several genetic studies of Tibetan hypoxia adaptation have recently found that some genes are different from those of the Han population. For example, hypoxia-related genes, EPAS1, SENP1, PPARGC1A and EGLN1, were suggested to be responsible for plateau adaptation in Tibetan (Beall et al. 2010; Zhou et al. 2013a; Bigham and Lee 2014; Lorenzo et al. 2014).

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Correspondence: Xiaofang Pei, West China School of Public Health, No.4 West China Teaching Hospital, Sichuan University, #16 Section 3, Renmin Nan Lu, Chengdu, Sichuan 610041, China.
e-mail: xxpeiscu@163.com
Haojiang Zuo, West China School of Public Health, No.4 West China Teaching Hospital, Sichuan University, #16 Section 3, Renmin Nan Lu, Chengdu, Sichuan 610041, China.
e-mail: zuohaojiang@scu.edu.cn

Tibetan also developed unique lifestyles, cultures and dietary habits. And as for the diet structure, meat (beef and mutton), yak butter, milk, and other dairy products are their primary food sources, while vegetables and fruits consumption are significantly less than that in the Han population living in low altitude (Rong et al. 2012; Zhiyang 2012). However, whether similar genetic changes and specific diets affect microbiota in human is not clear.

The microbes in our bodies collectively make up to 100 trillion cells, and a growing body of evidence suggests that the composition and function of microbiota play vital roles in human development, physiology, immunity, and nutrition (Ley et al. 2006). The gut microbiota can directly influence human health and show adaptive potential to different lifestyles (Charbonneau et al. 2016). The oral cavity, a human-associated microbial habitat, also harbors much microbiota that plays essential health roles. And the oral microbiota also has the potential to influence gut and overall health. In the past 40 years, more than 250 oral species have been isolated and characterized by cultivation, and over 450 species have been identified by culture-independent molecular approaches (Aas et al. 2005; Paster et al. 2006). Both fungi and bacteria residing in the oral cavity are critical components of health and disease. Emerging evidence suggests that the oral microbiota is closely related to oral diseases, including periodontitis and dental caries (Willis and Gabaldon 2020), and may be associated with systemic diseases, including diabetes (Ohlrich et al. 2010), cardiovascular disease (Koren et al. 2011) and several cancers (Fan et al. 2018; Hayes et al. 2018). Data from these studies suggested that the oral microbiota may be crucial to the human host health or disease status, however, little is known about the overall structure of microbiota from the oral cavities of healthy Chinese plateau and plain populations.

It has been demonstrated that a close relationship exists between oral microbiota and various health problems. Different geographic origins of humans may result in diverse oral microbiota compositions due to distinctive life environments, genetic background, dietary habits, medical treatment, and other factors (Tremaroli and Backhed 2012). However, a comparison of oral microbiota in groups living at different altitudes has not been studied. Little is known about the correlations between the composition of oral microbiota and environmental factors, genetic backgrounds, lifestyle characteristics, and dietary habits of the Tibetan and Han population at different altitudes. Hence, in this study, we first performed a comparative analysis of the relative abundances of various micro-organisms in the oral microbiota of Tibetan living at a high-altitude of 3,500 m and Han populations living at a low-altitude of 500 m by using next-generation sequencing (NGS) technology and associated bioinformatics tools. This study will provide new ideas for further research on these factors driving the composition of oral microbiota and mechanistic studies for plateau adaptation.

Materials and Methods

Ethics statement

According to the Helsinki Declaration's ethical guidelines, the experimental protocol was established and approved by the Medical Ethics Committee of Sichuan University (K2016038). Written informed consent was obtained from the individual participants. All experiments were performed in accordance with approved policies and regulations.

Sample collection

We enrolled 90 healthy native Tibetan living at a high-altitude of 3,500 m at least five years (Aba, Hongyuan, Sichuan Province) and 35 healthy Han adults who lived in the Chinese hinterland at a low-altitude of about 500 m at least five years (Chengdu, Sichuan province). All of the enrolled subjects were with a mean age of 59.04 ± 5.90 years old (mean ± SD) (range 50-70 years old). All subjects signed informed consent and had not used any antibiotics one month before sampling. Before collection, subjects refrained from drinking and eating for at least 10 hours. Five mL saliva was collected by allowing saliva to accumulate on the mouth floor followed by spitting into a specimen tube. Saliva containing sputum is not collected. After the saliva collection, samples were divided into two tubes on the ice tray within 1 hour, and finally were transported to our laboratory and stored at −80°C.

DNA extraction

Total microbial genomic DNA was extracted from saliva samples by using the E.Z.N.A. Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA), following the manufacturer's instructions, and stored at −20°C prior to further analysis. The quantity and quality of extracted DNAs were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and DNAs with an A260/280 ratio of 1.8-2.0 was used for subsequent PCR amplification. Integrity and size of DNA were checked by 1% (w/v) agarose gel electrophoresis.

16S rRNA gene and ITS1 gene amplicon sequencing

The V3-V4 region (338F-806R) of 16S rRNA was amplified by PCR with the forward primer 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and the reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′). PCR amplification of the fungi ITS1 region was performed using the forward primer (5′-GGAAGTAAAAGTCGTAACA-AGG-3′) and the reverse primer (5′-GCTGCGTTCTTCATCGATGC-3′) Sample-specific 7-bp barcodes were incorporated into the primers for multiplex sequencing. The PCR components were as follows: 5 × Q5 reaction buffer (5 μL), 5 × Q5 High-Fidelity GC buffer (5 μL), Q5 High-Fidelity DNA Polymerase (0.25 μL), 2.5 mM dNTPs (2 μL), 10 μM of each Forward and Reverse primer (1 μL), DNA Template (2 μL), and 8.75 μl of ddH_2O. The total

volume of the reaction was 25 μL. Thermal cycling consisted of initial denaturation at 98°C for 2 min, followed by 25 cycles consisting of denaturation at 98°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, with a final extension of 5 min at 72°C. PCR amplicons were purified with Agencourt AMPure beads (Beckman Coulter, Indianapolis, IN, USA) and quantified using the PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). The PCR products were performed using the Illlumina NovaSeq platform with MiSeq Reagent Kit v3 at Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China).

Data and statistical analysis

All these sequencing data were calculated with QIIME (V1.8.0) and visualized with R packages (v3.2.0). Briefly, raw reads with exact matches to the barcodes were assigned to respective samples and identified as valid sequences, and the low-quality reads were filtered. Paired-end reads were assembled using FLASH. After chimera detection, UCLUST (Edgar 2010) clustered the remaining high-quality sequences into operational taxonomic units (OTUs) at 97% sequence identity. A representative sequence was selected from each OTU using default parameters. OTU taxonomic classification was conducted by BLAST searching the representative sequences set against the Greengenes Database using the best hit. And an OTU table was further generated to record the abundance of each OTU in each sample and the taxonomy of these OTUs.

Rarefaction curve analysis was used to judge whether the sequencing data were adequate to characterize all the samples' bacterial richness. OTU-level α-diversity, such as Chao1, ACE, Sob, Shannon, and Simpson indexes, were calculated using the OTU table in QIIME. Two indexes were selected to identify community richness, namely, the Chao1 ACE and Sob indexes. The β-diversity was performed to investigate microbial communities' structural variation across samples using Bray–Curtis dissimilarity and visualized by principal coordinate analysis (PCoA). Analysis of similarity (ANOSIM) testing was performed to determine group similarities among saliva fungal and bacterial community structures (Peng et al. 2019). Taxa abundances at the phylum, class, order, family, genus and species levels were statistically compared among samples or groups by Metastats, and visualized as a boxplot. Linear discriminant analysis (LDA) with effect size measurement (LefSe) was used to explore discriminatorily abundant taxonomic characteristics between Tibetan (HY) and Han population (CD) using the Wilcoxon rank-sum test. Microbial taxa with LDA scores > 2.0 and a $P < 0.05$ were considered significantly different.

The PICRUSt approach was conducted to characterize the salivary microbiome's functional alterations between the Tibetan and Han population (Douglas et al. 2020). After that, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway abundances derived from the predicted KEGG ORTHOLOGY (KO) abundances were performed with MinPath (Kim et al. 2019; Kolbe et al. 2019). The predicted KOs were grouped into the levels of categorization, hierarchical levels 1, 2 and 3. Functional information in the form of guild assignment to fungal OTUs was performed using the online version of FUNGuild. FUNGuild parses OTUs into "guilds" or "functional groupings" based on their taxonomic assignments (Nguyen et al. 2016). Significant differences in KEGG categories and FUNGuild between two groups were determined using Welch's t-test. *P*-values were corrected for false discovery rate (FDR). Adjusted P -value < 0.05 was considered significantly different.

Results

Characteristics of sequencing

From the 16S rRNA and ITS gene sequencing data of the final 125 saliva samples (90 native Tibetan living in Hongyuan and 35 Han individuals living in Chengdu), We generated 16S rRNA datasets consisting of 13,968,666 filtered high-quality sequences, with an average of 111,749 sequences for each sample, and generated ITS datasets consisting of 12,925,091 filtered high-quality sequences, with an average of 103,400 sequences for each sample. All sequences were clustered at a 97% similarity of operational taxonomic units (OTUs). The maximum number of bacterial OTUs varied between 766 and 2,227, whereas the maximum number of fungal OTUs varied between 106 and 659. All samples' rarefaction curve already reached a plateau at this sequencing depth, suggesting that the sequencing was deep enough. These overlapping OTUs (985 OTUs in fungi and 3,554 OTUs in bacteria) were shared by the Tibetan and Han population (Fig. 1 A, B), which indicated that a core microbiome might exist in a healthy Chinese population.

Alpha diversity analysis of salivary microbiota

The alpha diversity of salivary flora was analyzed by Wilcoxon rank-sum test. As shown in Table 1, the Chao and ACE index of the Tibetan in fungi and bacteria were significantly higher than those in the Han Plain population ($P < 0.001$). Another abundance index Sobs also showed the same result ($P < 0.01$), suggesting that the abundance of fungi and bacteria in Tibetan was significantly reduced. Besides, in the analysis of community diversity, the Simpson index of the Tibetan is higher than that of the Han ($P < 0.01$), but the Shannon index of the Han population is higher than that of the Tibetan ($P < 0.05$).

Taxonomy-based comparisons of salivary microbiota

The OTU species classification was carried out by comparative analysis with the Greengenes database. Overall microbiota compositions for each group at the phylum and genus levels are shown in Fig. 1. In both Tibetan and Han groups, fungi's dominant phyla were *Ascomycota* and *Basidiomycota*, accounting for more than 90% of all

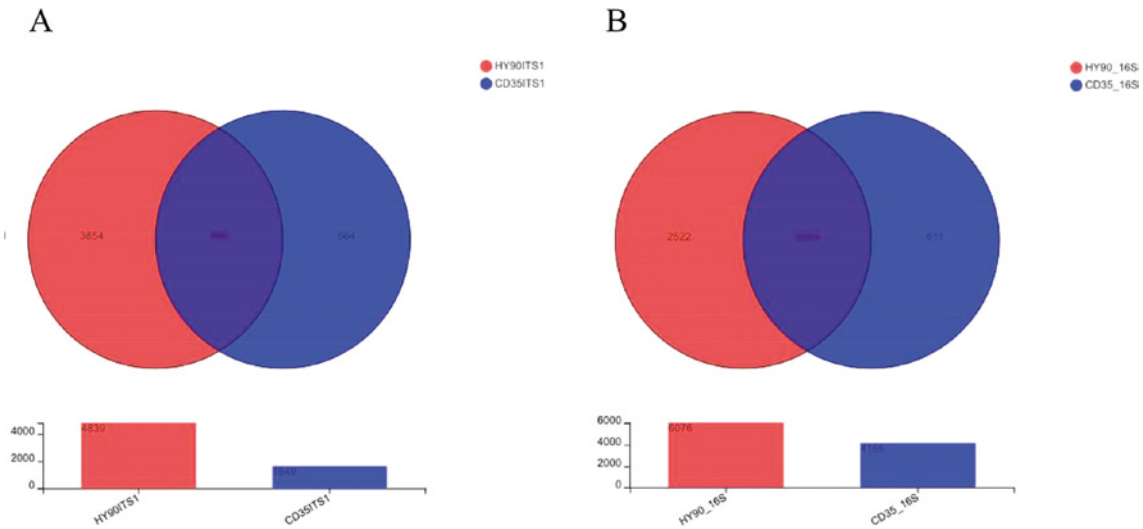

Fig. 1. Venn diagram.
A: Fungi. B: Bacteria.

Table 1. Analysis of α-diversity of salivary microbiota in Tibetan and Han.

	ITS (n = 125)		*P*-value	16sRNA (n = 125)		*P*-value
	HY (n = 90)	CD (n = 35)		HY (n = 90)	CD (n = 35)	
Chao	515.75 ± 82.65	276.10 ± 79.70	9.93e-17***	2023.10 ± 302.81	1819.20 ±173.76	1.96e-05***
ACe	711.8 ± 131.63	392.7 ± 116.13	8.86e-16***	2071.60 ± 280.76	1828.30 ± 179.99	1.06e-06***
Sob	296.49 ± 52.30	159.46 ± 46.31	4.91e-16***	1563.30 ± 282.63	1436.30 ± 158.38	0.004**
Shannon	2.06 ± 0.76	2.85 ± 0.89	7.58e-07***	4.21 ± 0.43	4.38 ± 0.33	0.04*
Simpson	0.30 ± 0.22	0.16 ± 0.18	1.061e-06***	0.07 ± 0.04	0.05 ± 0.02	0.008**

ITS, Internal Transcribed Spacer; HY, Tibetans living in Hongyuan; CD, Chinese Han living in Chengdu.
The richness estimator Chao, ACe and Sob index, diversity estimator Shannon and Simpson index were calculated at the 97% similarity level. Data were shown as mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

species (Fig. 2A). The relative abundance of *Ascomycota* in Tibetan was higher than that in the Han population ($P < 0.001$), whereas the *Basidiomycota* abundance appeared to be higher in the Han populations ($P < 0.001$). And the five dominant phyla of bacteria were *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Fusobacteria*. The relative abundance of *Streptococcus* and *Veillonella* in Tibetan was higher than that in the Han population ($P < 0.01$). In contrast, the *Porphyromonas* and *Neisseria* abundance appeared to be higher in the Han population ($P < 0.001$).

Relative abundances of major genera detected and statistical analyses among both groups are shown in Fig. 2B. At the genus level of fungi, the relative abundances of 5 genera were significantly higher in the HY group ($P < 0.001$), including *Candida*, *Fusarium*, *Zopfiella*, *Basidiomycota* and *Cordycipitaceae*. Surprisingly, the *Zopfiella* was found almost exclusively in the Tibetan. On the other hand, *Aspergillus*, *Malassezia*, *Peniophora*, *Cladosporium* and *Verticillium* were significantly lower in the HY group ($P < 0.01$). And the relative abundance of *Streptococcus*, *Veillonella* and *Rothia* in Tibetan were higher than that in the Han population ($P < 0.05$). In contrast, the *Porphyromonas* and *Neisseria* abundance appeared to be higher in the Han populations ($P < 0.001$).

Beta diversity analysis

To further analyze whether the salivary microbiota structure differs between native Tibetan and the Han population, Principal Coordinate Analysis (PCoA) based on the relative abundance of OTUs was performed. The salivary fungi results revealed significant differences in salivary microbial community structure between the Tibetan and the Han population regarding the first two principal component scores, which accounted for 22.34% and 16.98% of the total variations. And the results of salivary bacteria of the Tibetan and Han population also showed significant separation between the two groups. PC1 and PC2 accounted for 20% and 13.98% of the total variations, respectively (Fig. 3).

Differences in salivary microbiota between Tibetan and

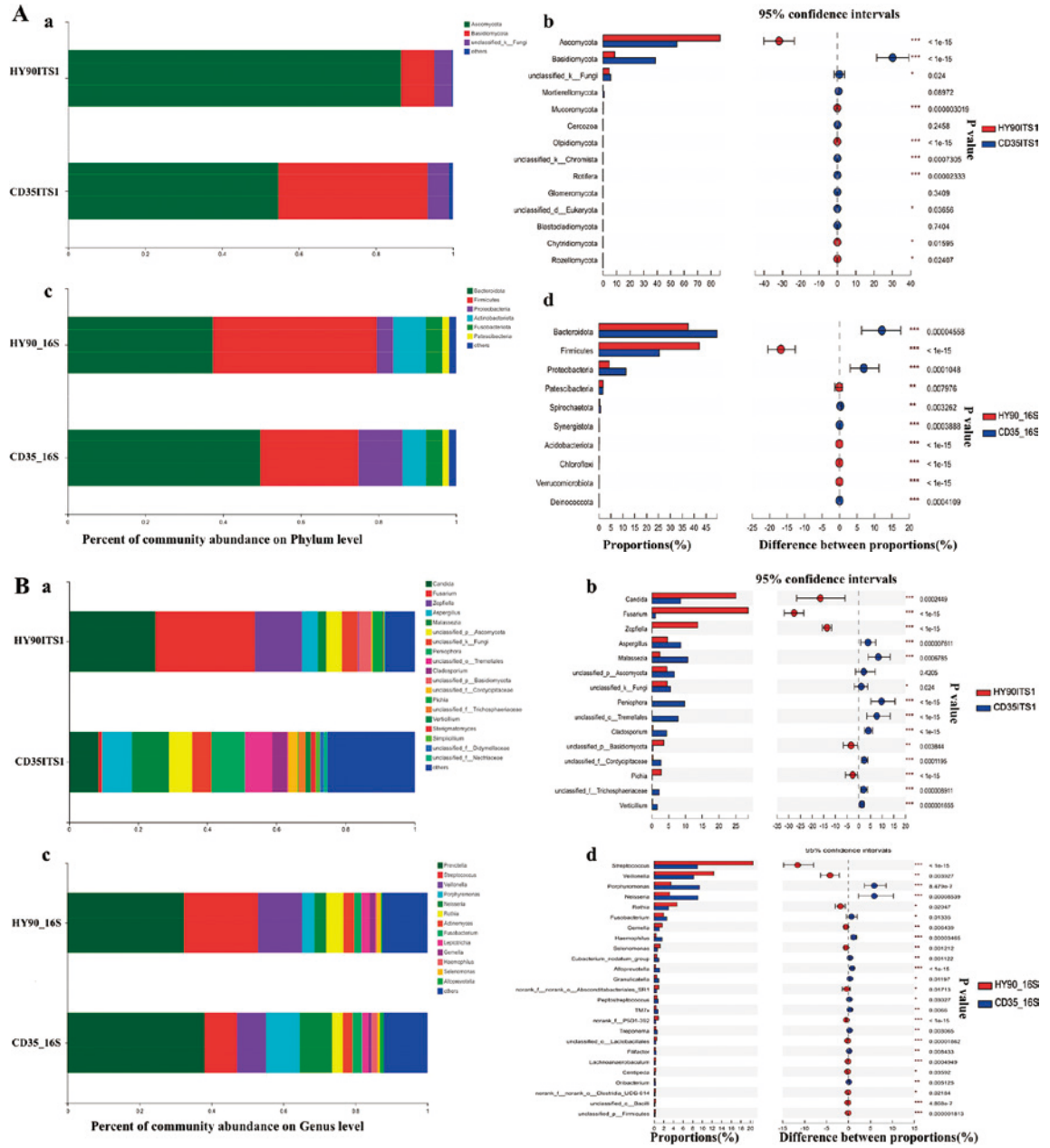

Fig. 2. Compositional differences in the salivary microbiota at the Phylum (A) and Genus (B) level. Relative fungial (A, a~b; B, a~b) and bacterial (A, c~d; B, c~d) abundance in the salivary microbiota of the Tibetan and Han populations. Wilcoxon rank-sum test methods calculated *P* value. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Han populations

The differences in salivary microbiota between the two groups were determined by both linear discriminant analysis effect size (LEfSe) [the logarithm of linear discriminate analysis (LDA) > 2] and the Wilcoxon test ($P < 0.05$). The common results of the two methods were considered to show differentially abundant fungi and bacteria. Compared with that in Tibetan, the relative abundances of *Basidiomycota*, *Agaricomycetes*, *Russulales*, *Peniophoraceae Malasseziales* were higher in the Han population. The relative abundances of *Ascomycota, Sordariomycetes, Fusarium, Nectriaceae* and *Hypocreales* were higher in Tibetan. The same analysis was performed for two groups of bacteria abundance. In Tibetan, the relative abundances of *Bacteroidales*, *Bacteroidia*, *Gammaproteobacteria* and *Proteobacteria* were decreased than those in the Han population. But more *Firmicutes*, *Bacilli*, *Lactobacillales* and *Streptococcaceae* were founded in Tibetan (Fig. 4).

PICRUSt and FUNGuild functional prediction analysis

Bacterial and fungal function profiles between HY and CD group were generated using the KEGG pathway database and the FUNGuild database. The KEGG pathways

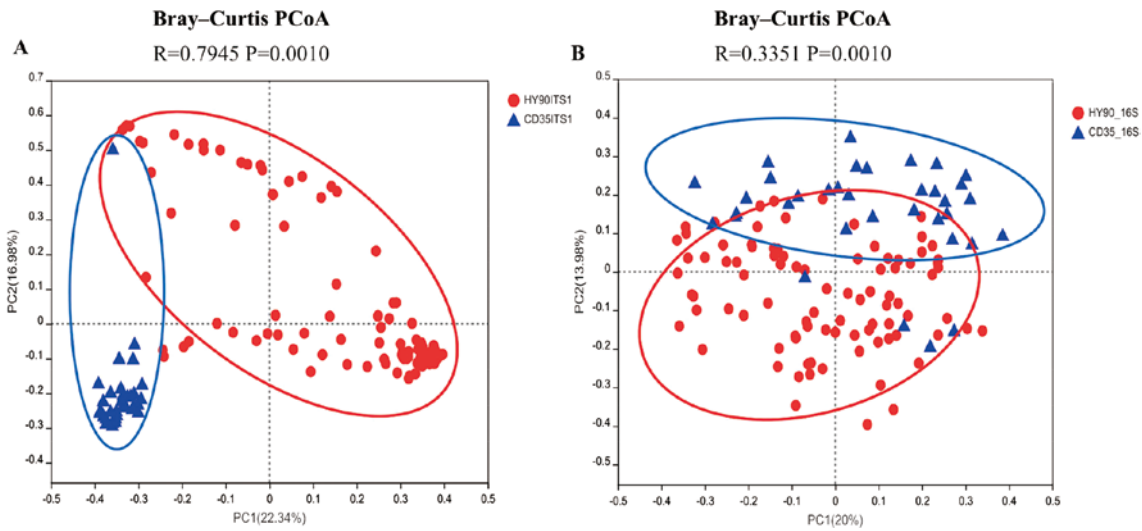

Fig. 3. Principal coordinates analysis (PCoA) on the distance matrix of Bray-Curtis between Tibetan and Han population.
A: Fungi. B: Bacteria.
P-values were determined from 999 permutations in the analysis of similarity test (ANOSIM).

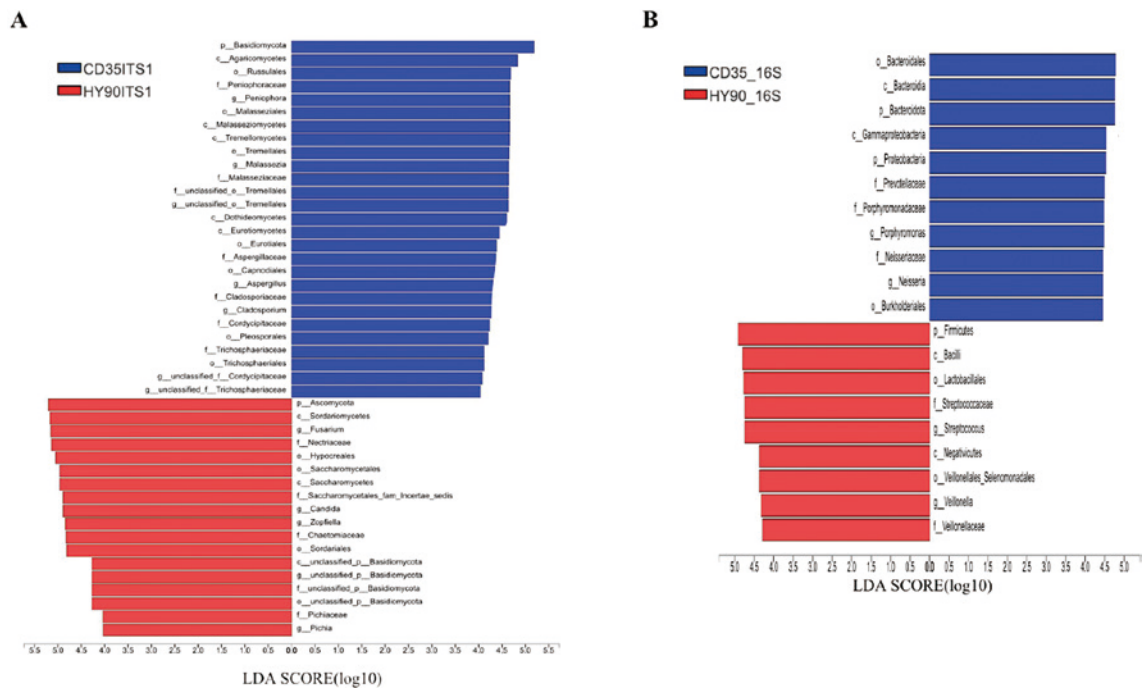

Fig. 4. LEfSe analysis between Tibetan and Han population.
Lefse software [linear discriminant analysis (LDA) coupled with effect size measurements] was used. LefSe showed a list of specific oral fungi (A) and bacteria (B) that enable discrimination between the Tibetan and Han population. Taxa enriched in the Tibetan population are indicated with a positive LDA score (green), and taxa enriched in the Han population have a negative score (red). Only taxa meeting an LDA significant threshold of 4 are shown. For taxa, which were defined as unclassified or Incertae-Sedis, the name of a higher taxon level was added before its taxon abbreviation.
p, phylum; c, class; o, order; f. family; g, genus; s, species.

database classified biological metabolic pathways into six categories (KEGG level 1), including metabolism, genetic information processing, environmental information processing, cellular processes, organ systems, and human diseases. Among these pathways, metabolism, genetic information processing, environmental information processing, and organ systems were significantly different between the two groups (Fig. 5). Also, the KEGG level 2 and level 3 functional profiles were also compared between the two groups, we found that the significantly enriched function categories in HY group were related to nervous system, lipid metabolism, membrane transport and signal transduction (Table 2).

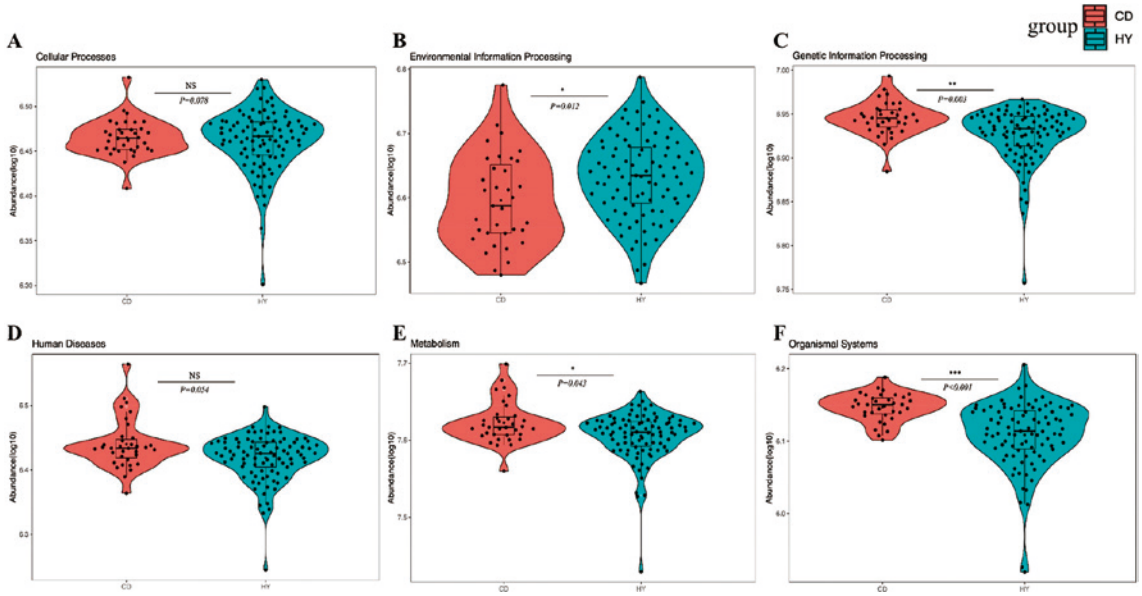

Fig. 5. Comparison of PICRUSt predicted KEGG function data based on Tibetan and Han population. Violin plots of (A) Cellular Processes, (B) Environmental Information Processing, (C) Genetic Information Processing, (D) Human Disease, (E) Metabolism, (F) Organismal Systems. FDR multiple comparison correction methods calculated adjust *P*-value. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; NS, Not Significant.

Table 2. PICRUSt predicted functions of Kyoto Encyclopedia of Genes and Genomes (KEGG) categories represented in the comparison between CD group and HY group.

KO functional categories			Difference in the means between CD and HY (95% CI)[a]	Adjusted *P*-values[b]
Level 1	Level 2	Level 3		
Organismal Systems	Immune system	NOD-like receptor signaling pathway	5.03e+03(1.98e+03~8.09e+03)	3.59e-03
		Antigen processing and presentation	7.13e+03(4.65e+03~9.60e+03)	1.68e-06
	Nervous system	Dopaminergic synapse	−9.33e+01(−1.12e+02~−7.44e+01)	1.12e-14
		Serotonergic synapse	−8.97e+01(−1.08e+02~−7.11e+01)	2.03e-14
		Retrograde endocannabinoid signaling	−3.54e+00(−4.26e+00~−2.81e+00)	1.53e-14
Metabolism	Carbohydrate metabolism	Glyoxylate and dicarboxylate metabolism	7.29e+04(4.53e+04~1.00e+05)	1.09e-05
		Butanoate metabolism	4.78e+04(3.02e+04~6.54e+04)	4.07e-06
	Energy metabolism	Oxidative phosphorylation	1.44e+05(9.36e+04~1.94e+05)	3.13e-06
		Carbon fixation pathways in prokaryotes	1.09e+05(7.73e+04~1.42e+05)	3.26e-08
	Metabolism of cofactors and vitamins	One carbon pool by folate	3.97e+04(2.55e+04~5.40e+04)	1.68e-06
		Folate biosynthesis	5.40e+04(3.46e+04~7.34e+04)	3.16e-06
		Nicotinate and nicotinamide metabolism	8.24e+04(6.45e+04~1.00e+05)	2.07e-12
	Metabolism of other amino acids	Glutathione metabolism	3.44e+04(1.57e+04~5.32e+04)	1.84e-03
		D-Glutamine and D-glutamate metabolism	4.44e+03(1.55e+03~7.32e+03)	6.03e-03
		Cyanoamino acid metabolism	1.16e+04(6.32e+03~1.69e+04)	1.36e-04
	Nucleotide metabolism	Purine metabolism	7.74e+04(3.28e+04~1.22e+05)	2.11e-03
		Pyrimidine metabolism	6.04e+04(2.60e+04~9.48e+04)	1.72e-03
	Lipid metabolism	Glycerolipid metabolism	−3.31e+04(−4.70e+0.4~1.91e+0.4)	4.55e-05
		Synthesis and degradation of ketone bodies	−1.37e+04(−1.73e+0.4~1.00e+0.4)	1.47e-09
environmental Information Processing	Membrane transport	Phosphotransferase system (PTS)	−1.11e+05(−1.49e+05~−7.35e+04)	7.99e-07
	Signal transduction	cAMP signaling pathway	−2.11e+01(-2.95e+01~−1.27e+01)	9.34e-06
Genetic Information Processing	Folding, sorting and degradation	RNA degradation	3.38e+04(2.14e+04~4.62e+04)	3.54e-06

KO, Kyoto Encyclopedia of Genes and Genomes Orthology; 95% CI, 95% Confidence Interval.
Only interested (adjusted $P < 0.05$) level 3 functions for CD vs HY groups were included in this table, and all adjusted *P* values are less than 0.01.
[a]Compared the difference in the means of abundance of functional trait between CD and HY groups.
[b]*P*-values were corrected for the false discovery rate (FDR).

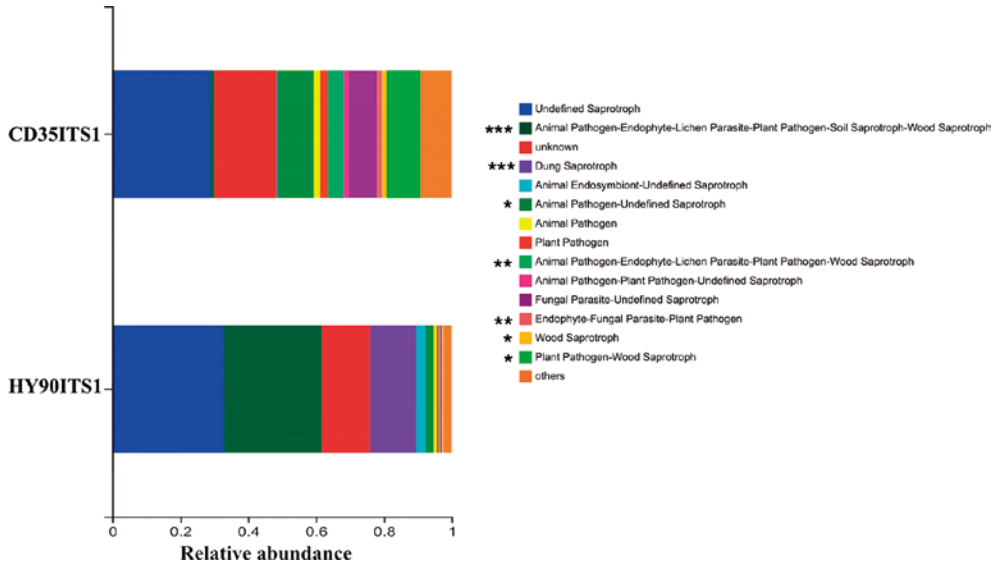

Fig. 6. The relative abundance between Tibetan and Han population assigned by FUNGuild for fungal communities. FDR multiple comparison correction methods calculated adjust *P*-value. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Notably, gene ontologies of essential metabolic pathways in the Metabolism category were present with a reasonable majority (https://docs.qq.com/sheet/DUm5xaWxsc1JQSlFK: Table S1). FUNGuild was used to predict the nutritional and functional groups of the fungal communities. The results showed that the pathotrophs, saprotrophs, and symbiotrophs were the major components (https://docs.qq.com/sheet/DUm5xaWxsc1JQSlFK: Table S2). The relative abundance of Animal Pathogen-Endophyte-Lichen Parasite-Plant Pathogen-Soil Saprotroph-Wood Saprotroph and Dung Saprotroph in the HY group were higher than the CD group, while Plant Pathogen-Wood Saprotroph and Endophyte-Fungal Parasite-Plant Pathogen in the HY group were significantly lower than that observed in the CD group (Fig. 6).

Discussion

Salivary microbiota is an integral part of our body's environment, yet the relative importance of environment, ethnic background, and diet to oral microbiome composition are still poorly understood. In this study, next-generation sequencing technology (NGS) was used to analyze the fungal and bacterial microbial diversity and community structure of the whole saliva from 90 native Tibetan living in Hongyuan and 35 Han individuals living in Chengdu. All of the participants had not taken antibiotics in the past month, circumventing the effects of antiviral drugs on microbiota. Overall, we found significant differences in the species composition of oral microbiota between the Tibetan and Han populations.

The microbiota analysis suggests that the structure and abundance of salivary microbiota in Tibetan and Han populations have significantly changed. The significant difference in either OTU abundance (Chao, ACE, Sob index) or OTU diversity index (Simpson, Shannon index) was observed between the Tibetan and Han populations. The overall taxonomic distribution of our data demonstrated that the richness of salivary microbiota from Han populations was more complicated than Tibetan. At the genus level, the main relative abundances of *Candida*, *Fusarium*, *Zopfiella*, *Streptococcus*, *Veillonella* and *Rothia* in Tibetan were higher than those in the Han population, whereas the *Aspergillus*, *Malassezia Porphyromonas* and *Neisseria* abundance appeared to be higher in the Han populations. Compared to the Tibetan, Hao et al. (2021) found that the Han population had higher relative abundances of Porphyromonas and Treponema genus organisms, especially Porphyromonas. Then, through LEfSe analysis, we also found that more *Ascomycota, Sordariomycetes, Fusarium Firmicutes*, *Bacilli*, *Lactobacillales* and *Streptococcaceae* were founded in Tibetan. Therefore, our results have revealed that different ethnic backgrounds differ in the composition of the oral microbiome.

The National Institute of Health's Human Microbiome Project (HMP) identified *Bacteroidetes*, *Actinomycetes*, *Firmicutes* and *Proteobacteria* as the dominant phyla accounting for over 95% of the entire oral microbiome (Human Microbiome Project Consoritium 2012; Zhou et al. 2013b). In our study, the oral bacteria of Tibetan and Han populations was consistent with HMP study results, indicating that the sequencing results were reliable. We also detected similar dominant species in HY and CD groups, including *Prevotella*, *Streptococcus*, *Neisseria*, *Veillonella*, and *Porphyromonas*. The sequence of dominant species can be different, which means that salivary microbiota's composition and structure may be different among diverse populations. These bacteria are considered to be closely related to human health. Among them, *Porphyromonas* is closely associated with the occurrence and development of periodontitis, and is also a driving factor for developing

digestive tract tumors, including colorectal cancer and pancreatic cancer (Duran-Pinedo and Frias-Lopez 2015). Karpinski (2019) reported that *Streptococcus* and *Prevotella* are closely related to oral cancer incidence. In addition, *Veillonella*, *Neisseria* and *Actinomyces* have been proven to be the dominant bacteria in the oral cavity of healthy people in many studies (Jiang et al. 2016; Meuric et al. 2017). These microorganisms are not pathogenic in a healthy state, but they may cause diseases that affect the host's health when the immune function of the body is reduced or the structure of oral microbiota is disordered. Due to their ubiquitous nature, the presence of these bacterial in healthy individuals' oral cavities was not surprising, which are most likely of environmental origin, from food and mouth breathing.

As reported previously, the human microbiome diversity is not limited only to bacteria but also includes fungal species. However, the oral microbiota was only recently discovered, and despite their potential great scientific importance, we found only a few studies where their composition was analyzed using high-throughput sequencing. Ghannoum et al. (2010) first determined the fungal microorganisms in healthy individuals' oral saliva by using 454 pyrosequencing and identified 74 cultivable fungal and 11 non-cultivable fungal genera. They found *Candida* species were the most frequent, followed by *Cladosporium*, *Aureobasidium*, *Aspergillus* and *Fusarium,* and their result has revealed significant interindividual variation. In our study, the oral fungal microorganisms of the Tibetan and Han population showed considerable intergroup differences, among which C*andida*, *Fusarium* and *Zopfiella* were the dominant bacteria groups in Tibetan. At the same time, *Aspergillus*, *Malassezia* and *Candida* were the dominant bacteria groups in the Han population. Surprisingly, the *Zopfiella* was found almost exclusively in the Tibetan. There are studies which show that *Zopfiella* is found mainly in Marine and soil environments. In healthy individuals, the pathogenicity of these fungi may be controlled by other fungi in the oral microbiota, as well as the functional immune system. The first evidence of interactions among members of the oral fungal community and their association with specific diseases comes from a study that describes the characteristics of oral fungal communities in HIV patients. The authors found that a decrease in abundance of an indigenous fungus *Pichia* in uninfected individuals went hand in hand with an increase in *Candida's* mass (Mukherjee et al. 2014). Moreover, *Malassezia* species, previously described as commensals and pathogens of the skin and lungs, have been recently found as predominant commensals in saliva (Saunders et al. 2012; Dupuy et al. 2014). At the same time, more extensive sampling and longitudinal studies are needed to investigate whether the *Zopfiella* is the endemic to Tibetan or plateau populations.

PICRUSt analysis can predict the metabolic function of bacterial communities with high reliability (Langille et al. 2013). In this study, we classified the bacteria between two groups according to the KEGG pathways. The difference in predicted functional sequences between HY and CD groups were distinct. The results showed that the oral bacteria of different populations primarily comprised 46 secondary functional layers, such as energy metabolism and carbohydrate metabolism, showing functional abundance. Xenobiotics biodegradation and metabolism and signal transduction significantly increased in HY group, and we found significant differences in 170 gene sequences between the two groups at the KEGG level 3. These results showed that there were great differences in the metabolic functions of oral bacteria in and Han populations living at different altitudes. In addition, we classified the fungi by their ecological guild and trophic mode. The FUNGuild results predicted that these fungi primarily corresponded to trophic mode, including saprotrophs, plant pathogens, pathotrophs, and the pathotrophic-saprotrophic-symbiotrophic. We found that the pathotrophs, saprotrophs, and symbiotrophs were the major components. In addition, we observed that Animal Pathogen-Endophyte-Lichen Parasite-Plant Pathogen-Soil Saprotroph-Wood Saprotroph and Dung Saprotroph played a major role in HY group, while Animal Pathogen-Endophyte-Lichen Parasite-Plant Pathogen-Wood Saprotroph, Animal Pathogen-Undefined Saprotroph, Endophyte-Fungal Parasite-Plant Pathogen, Plant Pathogen-Wood Saprotroph and Wood Saprotroph were dominated in CD group. These results indicate that there are significant differences in the function of oral fungal communities between two groups. In addition, we also found that most of the oral fungi have a variety of nutritional modes, which enable these fungi with complex life histories to adopt different survival strategies to cope with different living conditions, further revealing that oral fungi and hosts of different nationalities often have a dynamic and balanced cooperative relationship. All these functional changes of bacteria and fungi between HY and CD groups showed that microorganisms might develop an adaptive mechanism in the process of succession under the influence of the living environment, as reported in a previous study (Zhu et al. 2019). Because of the limitations of PICRUSt and FUNGuild functional prediction analysis, this study only preliminarily predicted the functions of related bacteria and fungi. Further validation should be performed in future studies using methods such as metagenomics to better understand the function of the oral bacterial and fungal community from different populations.

This study is a cross-sectional study and has some limitations. Firstly, the selected samples' specificity is not enough to eliminate the impact of lifestyle and eating habits; secondly, the selected population is middle-aged and elderly, which cannot represent the whole population. Therefore, in the follow-up study, we can eliminate the environmental (altitude) and diet bias by increasing the grouping, and expanding the target population's age range to improve the reliability of the results. It would be more meaningful to observe the succession of oral microbes in

different age groups, form a longitudinal cohort and increase the sample size.

In conclusion, to the best of our knowledge, this is the first report of differences in oral microbiota between Tibetan and Han populations living at different altitudes. This study compared the oral microbial community structure and diversity of the Tibetan population living in high-altitude areas and the Han population, obtaining two groups of dominant species at different levels, and providing baseline data on the oral microbiota of different populations. Our results showed that the diversity of oral microbiota in the Tibetan population was higher than that in the Han population, indicating that there were fewer species and more similar composition in saliva of Han population. In contrast, the structure of saliva microbiota in the Tibetan population was more diverse and complex. The PICRUSt and FUNGuild analysis also indicated that the function of bacterial and fungal communities was altered between the two groups. The main reason for this difference may be due to different genetic backgrounds or living environments, and related to factors such as the oral health and diet habits. Our findings also may provide some insights for further study of oral microbiota dysbiosis-related diseases in Tibetan and Han populations.

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Author Contributions

Xiaofang Pei and Haojiang Zuo contributed to the initial design of this experiment. Ke Dong, Tianli Zheng and Ji Yue performed the experiment. Haojiang Zuo, Kunpeng Wu, Weipeng Wang and Xun He analyzed the data. Ke Dong, Ruocheng Luo, Lan You, Jingjing Li and Zehui Hong prepared the manuscript of this publication.

Conflict of Interest

The authors declare no conflict of interest.

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