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Comparative analyses of fecal microbiota in Tibetan and Chinese Han living at low or high altitude by barcoded 454 pyrosequencing

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Knowledge about the impact of altitude and ethnicity on human gut microbiota is currently limited. In this study, fecal microbiota from 12 Tibetans (T group), 11 Chinese Han living in Tibet (HH group) and 12 Chinese Han living in Shaanxi province (LH group) were profiled by 454 pyrosequencing. Analysis of UniFrac principal coordinates showed significant structural changes in fecal microbiota among the three groups. There were significant differences in the composition of fecal microbiota among the three groups at phylum and genus levels. At the phylum level, the fecal samples of HH and T groups had higher relative abundances of *Firmicutes*, whereas the LH group had a higher relative abundance of *Bacteroidetes*. These changes at the phylum level reflected different dominant genus compositions. Compared with the LH group, changes of *Firmicutes* and *Bacteroidetes* were mainly due to a significant decrease of *Prevotella* in the HH group and were primarily attributable to significant decreases of *Bacteroides* and *Prevotella* as well as a significant increase of *Catenibacterium* in the T group. In conclusion, our results suggest that high altitude may contribute to shaping human gut microbiota. Genetic and dietary factors may also explain the different microbiota compositions between Tibetan and Chinese Han.

The microbes in our bodies collectively make up to 100 trillion cells and they encode 100-fold more unique genes than our own genome¹. The abundant and diverse microbiota in the gastrointestinal tract plays an important role in host physiology, including immunomodulation, maintenance of the gut barrier and involvement in nutrient metabolism². The functions of microbiota in the gastrointestinal tract are largely determined by the composition.

Different geographic origins of humans may result in diverse compositions of gut microbiota, due to distinctive life environments, genetic background or dietary habits. De Filippo *et al.* (2010)³ found that children living in rural Burkina Faso had a higher bacterial diversity and a lower Firmicutes/Bacteroidetes (F/B) ratio than those of Italian children. In addition, genera *Xylanibacter* and *Prevotella*, which are related to cellulose and xylan hydrolysis, were exclusively present in gut microbiota of Burkina Faso children³. Similar research performed by Lin *et al.* (2013)⁴ revealed that children living in Bangladesh showed a higher bacterial diversity and different community composition as compared to children living in the United States of America. An across-country survey performed by Yatsunenکو *et al.* (2012)⁵ also showed that there were significant differences in the phylogenetic composition of fecal microbiota among individuals living in three different countries (Venezuela, Malawi and United States of America) and the authors attributed the differences to different geographic origins and age. In contrast, Arumugam *et al.*

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Group	Reads	OTUs	Chao	Coverage	Shannon
LH	7066 ± 1086	1597 ± 152	4159 ± 568	0.85 ± 0.02	5.56 ± 0.44
HH	6225 ± 827	1450 ± 232	3670 ± 620	0.85 ± 0.03	5.67 ± 0.40
T	7492 ± 1232	1732 ± 252	4521 ± 758	0.84 ± 0.03	5.54 ± 0.50

Table 1. Summary of pyrosequencing data. The number of reads, OTUs, richness estimator Chao and Ace, diversity estimator Shannon were calculated at the 97% similarity level. LH = Chinese Han living in Shaanxi; HH = Chinese Han living in Tibet; HH = Tibetans.

(2011)⁶ found no significant structural changes of gut microbiota of subjects from 6 different countries and no effects of host properties such as body mass index, age, or gender on gut microbiota structure. The conflicting results from various studies highlight the necessity that a wider range of human living in different areas should be studied to characterize the global extent of human-associated microbial diversity^{4,7,8} and reflect the compounding effects of different life environment, genetic background and dietary habit. The dietary factor has been recognized to have a dominant role in different gut microbiota³. As for genetic background, Khachatryan *et al.* (2008)⁹ was the first to show that a gene (MEFV) mutation could affect gut microbiota in humans. Subsequently, Goodrich *et al.* (2014)¹⁰ compared microbiota in more than 1,000 fecal samples obtained from the TwinsUK population and identified many microbial taxa whose abundances were influenced by host genetics. In addition, environmental change also results in altered gut microbiota. For example, studies have shown that short exposure to high-altitude can alter the composition of gut microbiota^{11,12}.

Tibet is a typical high altitude area in China and has special environment conditions, such as low barometric pressure, low temperature, low humidity and high radiation. The population in Tibet is mainly composed of Tibetans and Chinese Han. Indigenous Tibetans have been living at high altitude for many years. The hypoxia of altitude (hypobaric hypoxia) would thus have exerted substantial evolutionary selection pressure. Tibetans adapt well to the hypoxia environment because of their different genotypes formed by long time natural selection, called genetic adaptation¹³. Recently, genetic studies conducted on the hypoxia adaptation of Tibetans have found differences in several genes compared with lowland Han^{14–16}. For example, hypoxia-related genes EPAS1 and EGLN1 were suggested to be responsible for genetic adaptation of high-altitude hypoxia in Tibetan^{14–17}. However, whether similar genetic changes have occurred to affect gut microbiota is not clear. Tibetans also have different diet habits, culture and lifestyles from Chinese Han^{18,19}. By using quantitative polymerase chain reaction (qPCR), Kowk *et al.* (2014)⁸ compared 10 major bacterial groups from the 4 dominant phyla of the common core gut microbiota between Chinese Han and Tibetans and found different fecal bacterial composition and a weak difference in structure. However, due to technical limitations, qPCR used by Kowk *et al.* (2014)⁸ is not suitable to study comprehensive changes of microbiota. Therefore, one objective of this study was to compare different ethnic origins (Han and Tibetan) on gut microbiota by 454 bar-code pyrosequencing.

Individuals from low-altitude populations (Han) suffer from a number of potential diseases specifically related to the low levels of oxygen, when they move to live at high altitude²⁰. Unlike Tibetan, Chinese Han who recently migrated and are living in Tibet may adapt to the hypoxia environment in different ways. A series of physiological changes may take place such as ventilation, cardiac function, oxygen delivery, muscle structure, oxygen consumption, and metabolism^{21,22}. Short exposure to high-altitude could affect the composition of gut microbiota^{11,12}. However, whether the physiological changes in the Chinese Han's acclimatization to high altitude are accompanied by changes of gut microbiota is still unknown. So another objective of this study was to investigate the effect of altitude on gut microbiota by comparing Chinese Han living at low altitude to those living at high altitude.

Results

Characteristics of pyrosequencing results. In total, 442,737 raw reads were obtained from all 35 fecal samples. After filtering, 294,698 high-quality sequences were produced, with an average of 6923 ± 1191 reads per sample. The total number of OTUs at the 97% similarity level was 22,913 (Table 1 and Table S1). The Shannon-Wiener curve of all samples already reached a plateau at this sequencing depth (Fig. 1), suggesting that the sequencing was deep enough. Good's coverage estimations revealed that 80.3% to 91.0% of the species were obtained in all of the samples. Estimators of the community richness (Chao) and the diversity (Shannon index) are summarized in Table 1. There were no statistically significant differences in the community richness estimator (Chao) and the diversity estimator (Shannon index) among three groups (Table 1).

Taxonomy-based comparisons of fecal microbiota at the phylum and genus levels among three groups. Overall microbiota compositions for each group at the phylum and class levels are shown in Fig. 2. There were 14 phyla and 21 classes in the fecal samples. The dominant phyla of all groups were *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* while the dominant classes of all groups were *Clostridia*, *Bacteroidia* and *Erysipelotrichi*. At the phylum level, the most predominant phylum was

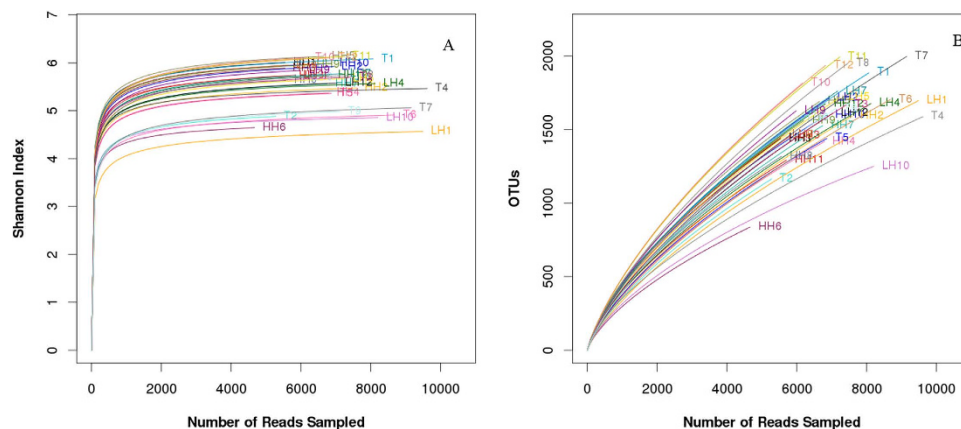


Figure 1. Rarefaction curves and Shannon-Wiener curves of each sample. Shannon-Wiener curves (A) and rarefaction curves (B) were all calculated at the 97% similarity level with pyrosequencing data in microbiota from groups of LH, HH and T. LH = Chinese Han living in Shaanxi; HH = Chinese Han living in Tibet; T = Tibetans.

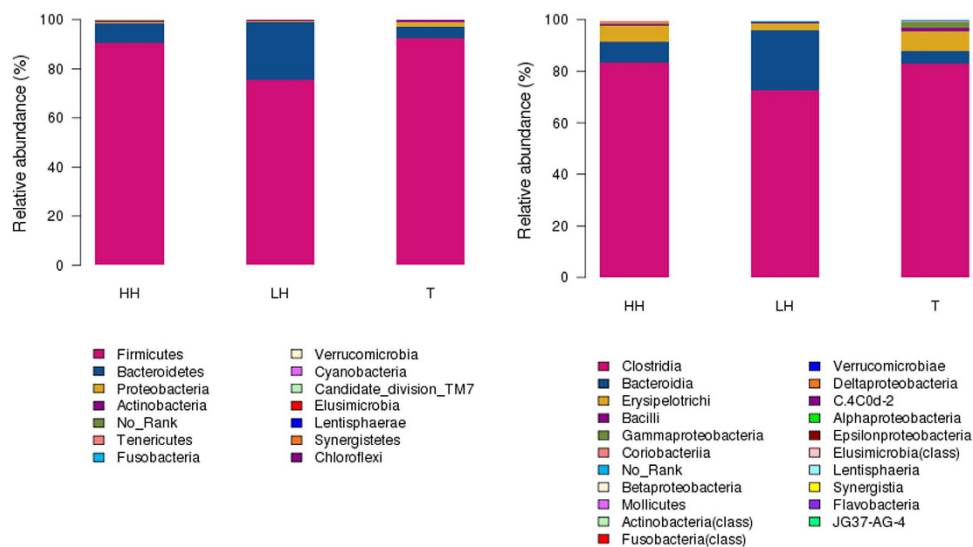


Figure 2. Relative abundance of bacterial phyla (A) and classes (B) in fecal microbiota of three groups. LH = Chinese Han living at the lowland; HH = Chinese Han living in Tibet; T = Tibetans.

Firmicutes, contributing 75.31%, 90.45% and 92.77% of the fecal microbiota in LH, HH and T groups respectively, followed by *Bacteroidetes*, contributing 23.59%, 7.98% and 4.69% respectively. The microbial composition varied greatly among individuals. Relative abundances of the phyla were analyzed by using a metastats test which either incorporates a paired t-test or uses a paired Wilcoxon rank sum test²³ and are shown in Table 2. *Firmicutes* was significantly more abundant in the fecal microbiota of HH and T groups than that in the LH group ($p = 0.022$ and 0.006 , respectively), while *Bacteroidetes* was significantly less abundant in the fecal microbiota of HH and T groups than that in the LH group ($p = 0.017$ and 0.001 , respectively). *Actinobacteria* differed between LH and HH groups ($p = 0.044$). *Cyanobacteria* and *Fusobacteria* exhibited significant differences between HH and T groups ($p = 0.042$ and 0.037 , respectively), while *Verrucomicrobia* differed between LH and T groups ($p = 0.027$).

Relative abundances of major genera detected and statistical analyses among three groups are shown in Fig. 3. There were 120 genera in fecal samples. At the genus level, the relative abundances of 13 genera were significantly higher in the HH group than in the LH group ($p < 0.05$), including *Acidaminococcus*, *Actinomyces*, *Blautia*, *Butyricimonas*, *Clostridium*, *Desulfovibrio*, *Helicobacter*, *Leuconostoc*, *Peptostreptococcaceae Incertae Sedis*, *Prevotellaceae uncultured*, *Prevotella*, *RC9 gut group* and *Rhodococcus*. On the other hand, 3 genera were significantly lower in the HH group than in the LH group ($p < 0.05$), including *Butyricimonas*, *Oscillospira* and *Sutterella*. The relative abundances of 16 genera were significantly higher in HH group than in T group ($p < 0.05$), including *Actinomyces*, *Alistipes*,

Phylum	Relative abundance (%)			Significance		
	LH	HH	T	LHvsHH	LHvsT	HHvsT
Firmicutes	75.311	90.453	92.770	*	**	NS
Bacteroidetes	23.591	7.983	4.690	*	**	NS
Actinobacteria	0.802	0.210	0.616	*	NS	NS
Cyanobacteria	0.008	0.001	0.015	NS	NS	*
Fusobacteria	0.004	0.020	0.001	NS	NS	*
Verrucomicrobia	0.003	0.004	0.012	NS	*	NS

Table 2. Relative abundance at the phylum level and statistical significance among three groups. LH = Chinese Han living in Shaanxi; HH = Chinese Han living in Tibet; T = Tibetans. Significance: NS > 0.05, * ≤ 0.05, ** ≤ 0.01.

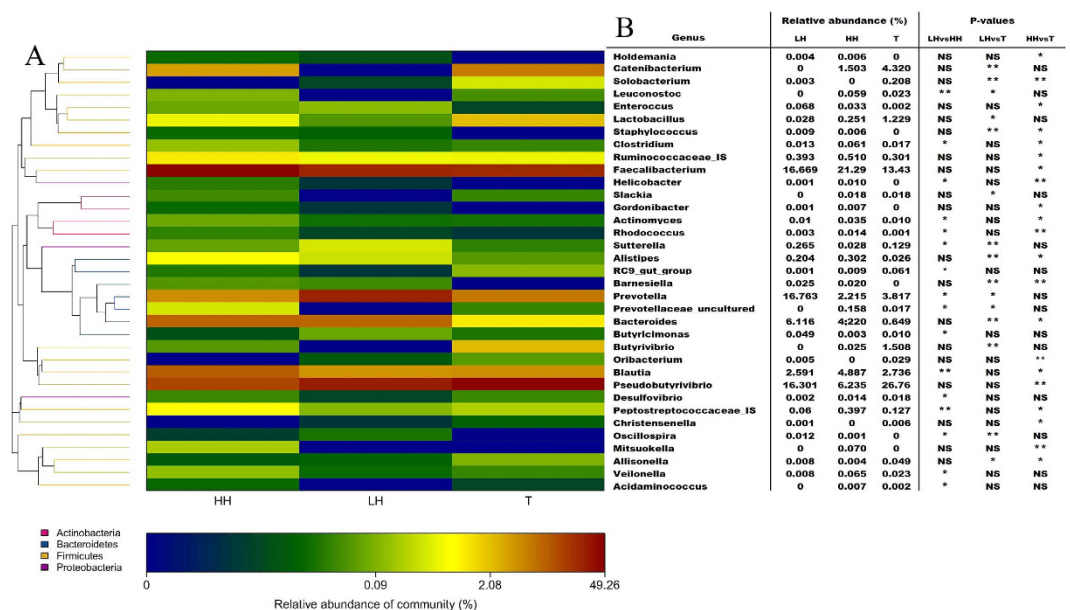


Figure 3. Heatmap of the distribution of the relative abundance of the genera. (A) Taxonomic classification of the genera. (B) Genera detected and statistical analysis among three groups (Metastats for the sequence count data). LH = Chinese Han living in Shaanxi; HH = Chinese Han living in Tibet; T = Tibetans. Significance: NS > 0.05, * ≤ 0.05; ** ≤ 0.01.

Bacteroides, *Barnesiella*, *Blautia*, *Clostridium*, *Enterococcus*, *Faecalibacterium*, *Gordonibacter*, *Helicobacter*, *Holdemania*, *Mitsuokella*, *Peptostreptococcaceae Incertae Sedis*, *Rhodococcus*, *Ruminococcaceae Incertae Sedis* and *Staphylococcus*. On the other hand, 5 genera were significantly lower in the HH group than in the T group ($p < 0.05$), including *Allisonella*, *Christensenella*, *Oribacterium*, *Pseudobutyrvibrio* and *Solobacterium*. The relative abundances of 6 genera were significantly higher in the LH group than in the T group ($p < 0.05$), including *Alistipe*, *Bacteroides*, *Barnesiella*, *Prevotella*, *Staphylococcus* and *Sutterella*. On the other hand, 8 genera were significantly lower in the LH group than in the T group ($p < 0.05$), including *Butyrivibrio*, *Catenibacterium*, *Lactobacillus*, *Leuconostoc*, *Oscillibacter*, *Prevotellaceae uncultured*, *Slackia* and *Solobacterium*.

Beta diversity of gut microbiota among three groups with multivariate statistics analysis. A weighted UniFrac principal coordinates analysis (PCoA) was performed to compare the overall structure of gut microbiota of all samples, based on the relative abundance of OTUs (at a 97% similarity level). There was an obvious separation of HH and T groups, while individuals were scattered for the LH group. Pco1, Pco2 and Pco3 accounted for 22%, 12% and 10% of the total variations, respectively (Fig. 4).

Other analysis methods (Jaccard, Bray-curtis and unweighted UniFrac) for the beta-diversity produced similar results (Figure S1).

Following the PCoA analysis, the statistical analyses showed that microbiota composition was significantly affected by ethnicity with various distance matrixes, but altitude significantly affected the microbiota composition only in weighted UniFrac, unweighted UniFrac and Abund_Jaccard analyses (Table S2).

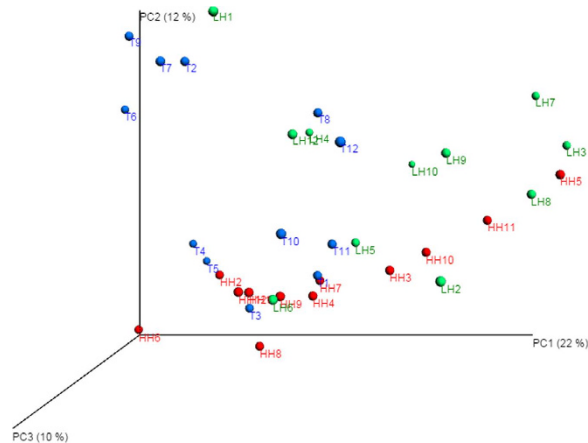


Figure 4. Weighted UniFrac principal coordinates analysis (PCoA) plots based on weighted UniFrac metric. LH = Chinese Han living in Shaanxi (green dots); HH = Chinese Han living in Tibet (red dots); T = Tibetans (blue dots).

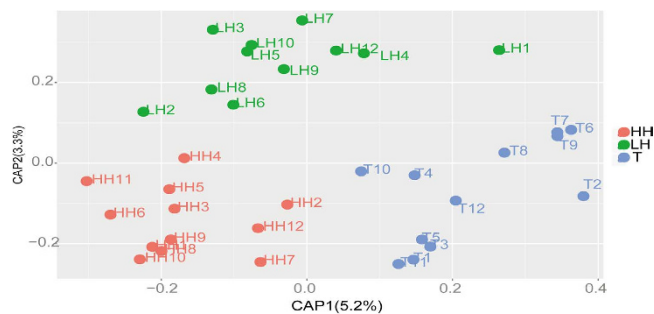


Figure 5. Canonical analysis of principal coordinates (CAP) of human gut microbiota with different ethnicity and altitude. CAP1 was the ethnic axis and CAP2 was the altitude axis. ANOVA test showed significant influence of the two constraints ($p = 0.001$). LH = Chinese Han living in Shaanxi; HH = Chinese Han living in Tibet; T = Tibetans.

These results are in general agreement with those from the canonical analysis of principle coordinates (CAP) (Fig. 5). However, ethnicity and altitude only accounted for 8.5% of the total variations, indicating that many other factors have effects on the microbiota composition among three groups.

Real-time qPCR. Analysis by real-time qPCR showed total bacterial numbers per 10 ng DNA significantly differed between LH and HH groups ($p = 0.023$). However, total bacterial numbers per 10 ng DNA did not differ between LH and T groups or between HH and T groups (Fig. 6A). Next, the prevalence of the two most abundant phyla of commensal bacteria in the gut, *Bacteroidetes* and *Firmicutes*, were assessed among the three groups. *Firmicutes* dominance was less in the LH group than in the HH and T groups ($p = 0.013$) (Fig. 6B). In contrast to the *Firmicutes* dominance, the *Bacteroidetes* dominance was more in the LH group than in the HH and T groups ($p = 0.021$) (Fig. 6C). Furthermore, the proportional representation of two phyla was significantly shifted in the HH and T groups, in favor of *Firmicutes* ($p = 0.017$) (Fig. 6D).

Discussion

Our results have provided additional evidence that the geographic origin contributes to shaping human gut microbiota, as previously reported^{3–5}. Our study focused on the differences of gut microbiota in human living at high altitude (Tibet) and low altitude (Shaanxi) as well as two ethnic groups (Tibetan and Chinese Han). Study subjects were carefully selected, such that indigenous Tibetans had been living in Tibet for generations, Chinese Han living at high altitude had been living in Tibet more than 4 years and Chinese Han living at low altitude never went to Tibet. Meantime, our participants were within narrow ranges of age (between 18 to 25 years old) and body mass index (BMI) (between 17.8 to 25.1) and all participants were males. Overall, we found different fecal microbiota compositions among the three groups, but no significant differences in the diversity.

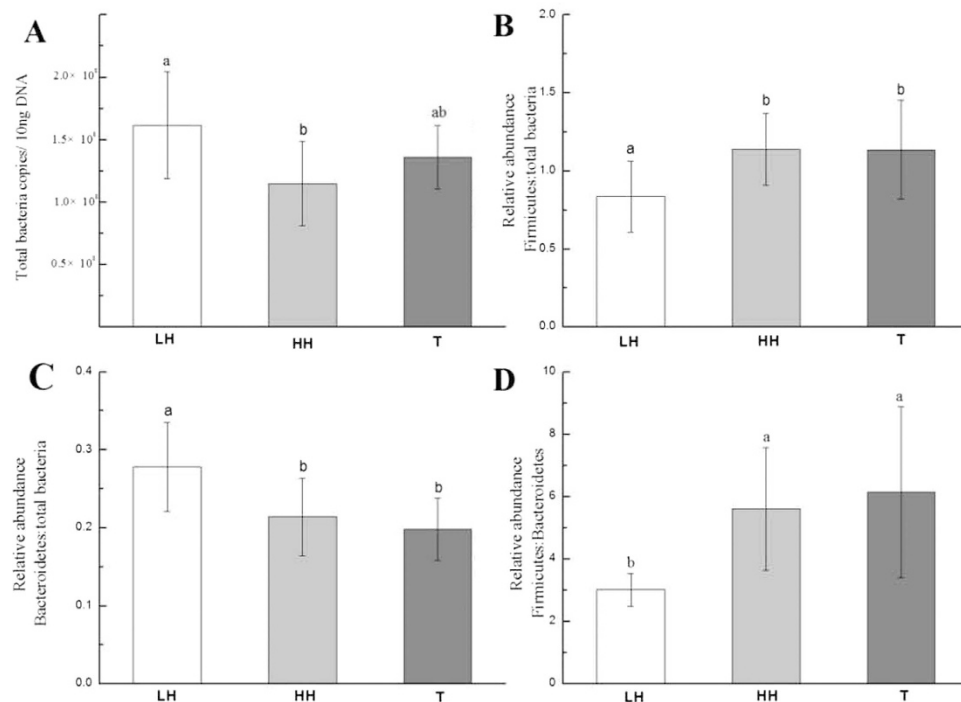


Figure 6. Relative abundance of total bacteria per 10 ng DNA (A), ratios of *Firmicutes*:total bacteria (B), ratios of *Bacteroidetes*:total bacteria (C), and ratios of *Firmicutes*:*Bacteroidetes* (D) in the fecal samples of three groups. ^{a,b} Different lower-case letters indicate significant differences between groups. LH = Chinese Han living in Shaanxi; HH = Chinese Han living in Tibet; T = Tibetans.

The gut microbiota of Tibetans was compositionally distinct from Chinese Han living at high or low altitude in this study. *Firmicutes* and *Bacteroidetes* are two prominent phyla in gut microbiota of humans and have received much attention. Our results showed that Tibetans had a high relative abundance of *Firmicutes* and a low relative abundance of *Bacteroidetes* compared with Chinese Han living at low altitude. However, there was no difference between Tibetans and Chinese Han living at the same high altitude area. These results from pyrosequencing were further verified by qPCR. Interestingly, these changes of the *Firmicutes* and *Bacteroidetes* at high altitude were similar to the shift observed in patients with obesity²⁴. However, most of the study subjects in our study had normal BMI according to the standards²⁵. In contrast, Kwok *et al.* (2014)⁸ found Chinese Han had a significantly higher F/B ratio than Tibetans. The high ratio of F/B was in response to a high-fat diet and did not affect energy harvesting²⁶. A previous large-scale survey revealed the existence of obvious differences in dietary intake between Chinese Han and Tibetans¹⁸, which was also observed in our study (Table S3). From the questionnaire we found that the frequency for intake of high fat foods such as buttered tea, cheese and meat for Tibetans was higher than that of the Han people. This may explain a high ratio of F/B in Tibetans compared with Chinese Han living in Shaanxi. Nevertheless, the higher ratio of F/B in the HH group than in the LH group could be caused by unknown factors other than high-fat diets, since the two groups of people had similar dietary habits (Table S3).

Compared with the LH group, the changes of the *Firmicutes* and *Bacteroidetes* in the T group were mainly due to significant decreases in genera *Bacteroides* (4.2% vs 0.6%) and *Prevotella* (16.7% vs 3.8%) and a significant increase in genus *Catenibacterium* (0% vs 4.3%). Furet *et al.* (2010)²⁷ found that *Bacteroides* and *Prevotella* were negatively correlated with energy intake. Tibetans have higher energy intakes than Chinese Han (13.7 MJ/d vs 11.1 MJ/d)¹⁸. This may explain low levels of *Bacteroides* and *Prevotella* observed in Tibetans. In addition, Tibetans had a high level of *Pseudobutyrvibrio* (26.8%). The metabolites of genus *Pseudobutyrvibrio* are mainly formate, butyrate and lactate²⁸, but the physiological function of *Pseudobutyrvibrio* as a part of the human microbiome is not yet known. While both T and HH groups had a high ratio of F/B, the HH group was mainly due to significant increases in genera *Faecalibacterium* (21.3% vs 13.4%) and *Bacteroides* (6.1% vs 0.6%) and a significant decrease in genus *Pseudobutyrvibrio* (6.2% vs 26.8%) in comparison with the T group. Several genome-wide studies confirmed that several genes showed significantly different genotype frequencies between Tibetans and Chinese Han^{14–16}. The gene differences can result in changing the composition of gut microbiota^{9,10}. Therefore, genetic and dietary factors may explain the differences in the composition of gut microbiota between Tibetans and Chinese Han living in the same high altitude area.

In order to further investigate the environmental factors at high altitude on compositions of gut microbiota, we also compared the gut microbiota between Chinese Han living at low altitude or at high altitude. The total bacteria of Chinese Han in the HH group were significantly decreased in comparison with the LH group. The decreased microbial load could weaken the intestinal ecosystem stability²⁹. This result suggests that the intestinal health of immigrants living in Tibet might be easily compromised. The HH group had a higher ratio of F/B than the LH group. These differences were mainly due to a significant decrease in *Prevotella* (2.2% vs 16.7%). *Prevotella* is associated with consumption of a diet rich in carbohydrates³⁰. However, Chinese Han living at low or high altitude have similar diet habits and genetic background. Therefore, environmental factors at high altitude are thought to have a certain effect on gut microbiota.

In our study, we found significant differences in genera *Clostridium*, *Desulfovibrio*, *Bacteroides*, *Lactobacillus* and *Prevotella* among the three groups. These genera of microbiota are all associated with production of short-chain fatty acids (SCFAs)^{3,31}. Humans living at high altitude have high energy demands³² and pulmonary hypertension³³. Metabolites produced by microbiota play important roles in host health by being involved in host metabolism³⁴. Microbiota can use non-digestible carbohydrates in the colon and produce SCFAs, mainly acetate, propionate and butyrate³⁴. SCFAs produced by the gut microbiota not only provide energy, but also decrease blood pressure via olfactory receptor 78 and G protein couple receptor 41³⁵. Different genera of microbiota can produce different metabolites. Although propionate exerts a hypotensive effect³⁴, the effects of different SCFAs produced by microbiota on blood pressure are still unknown. Our results suggest that gut microbiota potentially influences healthy status by modulating energy harvest and blood pressure response to hypoxia environment at high altitude.

In summary, our study revealed that humans living at high altitude (Tibetans and Chinese Han) had a high abundance of *Firmicutes* and a low abundance of *Bacteroidetes*. However, these differences were due to different genus compositions. Compared with the LH group, the changes of the *Firmicutes* and *Bacteroidetes* in the HH group were mainly due to a significant decrease in *Prevotella*. However, in the T group these changes were primarily attributable to significant decreases in *Bacteroides* and *Prevotella* and a significant increase in *Catenibacterium*. Whether these changes of genera could affect adaptation to high altitude needs further large-scale studies, taking socioeconomic status, sanitation and exercise intensity into consideration.

Experimental procedures

Sampling. We enrolled 12 healthy Tibetan adults (T group) and 12 healthy Han adults (HH group) living in Tibet (Nyingchi, Tibet, with an average altitude of 3100 meters above sea level) and 11 healthy Han adults (LH group) living in Shaanxi (Yangling, Shaanxi, with an average altitude of 450 meters above sea level) for this study. The individuals were all males within a narrow range of age (18–25 years old). They did not have bowel diseases or metabolic diseases and had not taken any antibiotics or probiotics in the three months before the sampling dates. All Tibetan participants were living in Tibet for generations and never left Tibet. Chinese Han in the HH group had lived in Tibet for more than 4 years. Detailed information of each participant is shown in Table 3. The fecal sample was collected from each subject. Fecal samples were frozen immediately after sampling and stored at -80°C . This study was approved by the Ethics Committee of Agricultural and Animal Husbandry College of Tibet University (Nyingchi, Tibet). Informed consent was provided by all individuals before participation. All experiments were performed in accordance with approved guidelines and regulations.

DNA extraction. Genomic DNA was extracted from fecal samples by using the E.Z.N.A. stool DNA Kit (Omega, USA) according to the manufacturer's instructions. The amount of DNA was determined by Nanodrop ND-2000 (Nanodrop, USA). Integrity and size of DNA were checked by 1% (w/v) agarose gel electrophoresis. All DNA samples were stored at -20°C until further processing.

Pyrosequencing. The V1-V3 region of the 16S ribosomal RNA (rRNA) gene from each DNA sample was amplified using the bacterial universal forward primer 27F: 5'-AGAGTTTGATCCTGGCTCAG-3' and the reverse primer 533R: 5'-TTACCGCGCTGCTGAC-3' containing the A and B sequencing adaptors. The cycling parameters were as follows: 2 min initial denaturation at 95°C ; 25 cycles of denaturation at 95°C (30 s), annealing at 55°C (30 s), elongation at 72°C (30 s); and final extension at 72°C for 5 min. Three separate PCR reactions of each sample were pooled for pyrosequencing. The PCR products were separated by 2% agarose gel electrophoresis and purified by using the AxyPrepDNA Gel extraction kit (Axygen, USA). Amplicon DNA concentrations were qualified by using a Quant-iTTM PicoGreen double-stranded DNA assay (Invitrogen, Germany). For sequencing, amplicons from each reaction were mixed in equal amounts based on concentrations and subjected to an emulsion PCR as recommended by 454 Life Sciences by using Roche GS FLX Titanium emPCR Kits (Roche, Switzerland). Using the Sequencing Method Manual XLR70 kit (Roche, Switzerland), sequencing was performed on the Roche Genome Sequencer GS-FLX Titanium platform by the Majorbio Bio-Pharm Technology (Shanghai, China).

Valid sequences were sorted into different samples according to barcodes. The programs SeqIn (<http://sourceforge.net/projects/seqclean>) and Mothur (http://www.mothur.org/wiki/Main_Page) were used for removing low quality sequences (i) that did not perfectly match the PCR primer at the beginning of

Sample ID ¹	Nationality	Age	Living in Tibet	BMI ²
HH1	Han	23	51 months	22
HH2	Han	22	51 months	19.3
HH3	Han	22	51 months	18.1
HH4	Han	23	51 months	21.5
HH5	Han	24	51 months	17.6
HH6	Han	23	51 months	20.9
HH7	Han	25	51 months	21.2
HH8	Han	22	51 months	18.7
HH9	Han	24	72 months	24.3
HH10	Han	21	60 months	18.7
HH11	Han	23	51 months	19.7
HH12	Han	22	49 months	20.3
T1	Tibetan	22	Always	17.8
T2	Tibetan	21	Always	18.2
T3	Tibetan	20	Always	18.6
T4	Tibetan	21	Always	19.5
T5	Tibetan	18	Always	22.2
T6	Tibetan	25	Always	23.1
T7	Tibetan	20	Always	22
T8	Tibetan	20	Always	20.8
T9	Tibetan	20	Always	20.3
T10	Tibetan	21	Always	20.2
T11	Tibetan	20	Always	21.5
T12	Tibetan	20	Always	19.4
LH1	Han	20	Never	25.1
LH2	Han	19	Never	24.9
LH3	Han	21	Never	22.7
LH4	Han	20	Never	21.9
LH5	Han	20	Never	18.4
LH6	Han	19	Never	18.6
LH7	Han	19	Never	21.3
LH8	Han	20	Never	18.2
LH9	Han	22	Never	24.2
LH10	Han	22	Never	23.6
LH12	Han	25	Never	22.3

Table 3. Basic information of experimental subjects. ¹LH = Chinese Han living in Shaanxi; HH = Chinese Han living in Tibet; HH = Tibetans. ²BMI = body mass index.

a read (allowing for 2 errors), (ii) that did not match a barcode and PCR primer at the end of a read (pairwise identity less than 95%), (iii) that were shorter than 200 base pair in length, (iv) that contained ambiguous and homologous nucleotides, or (v) sequence quality score was less than 25 (using minimum average quality score of 25, maximum number of N's = 0, minimum sequence length = 200).

The high-quality sequences were aligned in accordance with the SILVA alignment (<http://www.arb-silva.de/>) and similar sequences with a minimum pairwise identity of 97% were clustered into operational taxonomic units (OTUs) using the Mothur program (<http://www.mothur.org/wiki/Cluster>). OTUs that reached a 97% similarity level were used for diversity (Shannon), richness (Chao), Good's coverage, and rarefaction curve analyses using the Mothur program. Taxonomical assignments of OTUs exhibiting 97% similarity were performed using the Mothur (<http://www.mothur.org/wiki/Classify.seqs>) which uses a RDP Naive Bayesian classifier³⁶ to calculate the probability of a sequence from a given taxonomy in accordance with SSU rRNA database of SILVA 111 at an 80% confidence level. For each sample, the abundance of bacterial phyla was expressed as the percentage of total sequences. The beta-diversity was performed with QIIME v1.80 by choosing various distance measures³⁷. The Adonis test in the R package

“vegan” was conducted to test the significance of constrained factors on microbial community³⁸. The canonical analysis of principle coordinates (CAP)³⁹ was performed by the R package “vegan”. All plots were constructed using the R software. Testing for significantly different phyla and genus among three groups were performed using the Metastats software (<http://metastats.cbc.umd.edu/>). The number of permutations used to calculate the *P* value (significance threshold = 0.05, false discovery rate threshold = 0.2) was set to 1000.

Confirmation of pyrosequencing data by quantitative real-time PCR. In order to further confirm the pyrosequencing data, real-time qPCR reactions were performed as previously described⁴⁰ in a volume of 25 μ L containing 10 ng of fecal DNA, but the annealing temperature was changed to 60 °C. A plasmid containing a total bacteria, *Firmicutes* and *Bacteroidetes* 16S rRNA gene from a clone library was diluted from 1×10^3 to 1×10^9 (copies/ml) to construct standard curves for the detection of total bacteria, *Firmicutes* and *Bacteroidetes*. PCR reactions were performed with CFX96 system (Bio-rad, USA) using SYBR Premix Ex TaqII (Takara, Dalian, China). The data are represented as the mean \pm SD of triplicate qPCR values.

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Author Contributions

L.L. and X.Z. contributed to the initial design of this experiment. L.L. performed the experiment. L.L. and X.Z. analyzed the data. L.L. and X.Z. prepared the manuscript of this publication.

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