Characterization of gut microbial community of rhesus macaques under the high-altitude extreme environments

CURRENT STATUS: UNDER REVIEW

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DOI:
10.21203/rs.2.13400/v1

SUBJECT AREAS
General Microbiology

KEYWORDS
Rhesus macaque; gut microbial community; 16S rRNA gene; high-altitude environment
Abstract

Background: The mammal intestinal microbita involved in various physiological processes in host and play a key role in host environment adaption. However, for non-human primate (NHP), little is known about their gut microbial community in high-altitude extreme environment and much less to their adaption to high-altitude environment. In this study, we want to characterize gut microbial community of rhesus macaques from multiple high-altitude environment and by comparing it to low-altitude control group to reveal the differences between altitudes. Results: we collected the fecal samples of rhesus macaques from four high-altitude populations (above 3000m) and one low-altitude population (below 100m). We first analyzed the overlap of operational taxonomic units (OTUs) between populations and found 27.8% of OTUs (core OTUs) were shared by all five populations. The majority of these OTUs have a higher abundance, whereas the unique OTUs have a lower abundance. By calculating alpha diversity index, we found high-altitude populations exhibited higher diversity. Statistical analysis of beta diversity indicated there were significant difference between high and low altitude population. Significant difference in composition were detected at phylum and family. At phylum level, high-altitude gut microbial community were dominated by Firmicutes (63.7%), but low-altitude were dominated by Bacteroidetes (52.2%). At family level, high-altitude population were dominated by Ruminococcaceae (36.4%), but low-altitude were dominated by Prevotellaceae (43.9%). Additionally, the abundance of Christensenellaceae are significantly higher in all high-altitude populations (3.33%) than low-altitude population (0.77%), despite a low abundance in two altitudes. Finally, function prediction indicated there was a significant difference in gene copy number of 29 level-2 pathway between high and low altitude population; and 26 of them are higher in high-altitude, especially in membrane transport and carbohydrate metabolism. Conclusions: We found
the gut microbial community of high-altitude rhesus macaques is significantly distinct from low-altitude population in diversity, composition and function. High-altitude populations were dominated by Firmicutes and Ruminococcaceae, but low-altitude population by Bacteroidetes and Prevotellaceae. The difference in gut microbiota between two altitude macaque populations may be caused by different host diet, environmental temperature and oxygen pressure, and gut microbial microorganisms may play an critical role in adaptive evolution of rhesus macaques to high-altitude environment.

Background

The gastrointestinal tract of animal is habited by a complex microbial community, know collectively as the gut microbiota or intestinal microbiota. Over recent years, evidence has accumulated that the gut microbial community of animals involves in a wide ranging of processes in host including health, grow and development, behavior, even affect the nervous system by secreting hormone[1-11]. These microbiotas can also assist in the whole-body energy harvest[12]. For example, previous research has demonstrated ruminants depend heavily on gut microbiota to digest fiber in rumen, and panda without a rumen also relys on it’s microbiome to degrade hemicellulose and starch in bamboo to provide themselves with energy[13-16]. The functions of gut microbiota are mainly determined by their composition which is affected by many factors of the host, such as genetic background, health condition, environmental differences, and even social behavior[17-24]. Owing the importance and tight interaction relationship between gut microbiota and it’s host, microbiomes and it’s host could be considerated as a whole biological unit on which natural selection acts[25]. When faced with selection pressure, these biological unit could response from host or their gut microbiota composition or both[26-28].

The Tibetan Plateau (generally more than 3000m above sea level) has long been known as
the roof of the world and the forbidden area of life because of low oxygen content (40% lower than sea level), low temperature (even in warmest month most area mean temperature still below 10°C), great temperature difference, high ultraviolet and limited high-quality foods (eg. fruits and young leaves) which are rich in digestible carbohydrates, lipids and proteins. In total, high altitude habitat is inhospitable to most animals especially for these endothermic animals which have a higher metabolism rate and typically require much more oxygen and energy to sustain body temperature than ectothermic animals. Despite these extreme conditions, many creatures have colonized in the Tibetan Plateau. To adapt to harsh high-altitude environment, they have evolved in nuclear genes, mitochondrial genes, physiology, morphology and behavioral strategies[29-39]. Recently, a few of research give anew insight into high-altitude adaptation that gut microbiotas of herbivorous mammals may assist in this progress. High-altitude herbivorous animal like plateau pika, tibetan sheep and yak, their gut microbiota show a stronger fermentation ability and contain more genes in producing short-chain fatty acids(SCFAs) which provide energy to their host across epithelium, while their relatives in low-altitude contains more genes in producing methane[40, 41].

Non-human primates (NHPs) originated from a tropical rain forest habitat[30]. Most of NHPs prefer a warm habitat and distribute in tropical or subtropical environment including rain forest and savannah, only few spieces radiated into temperate forest[42]. Rhesus macaque(Macaca mulatta), a omnivorous NHPs, occupies an enormous range of habitats and climates, ranging from tropical rain forest through temperate forest even to palteau [43]. In addition, rhesus macaque is the most northern species of NHPs in the world. In China, rhesus macaque is the only NHP which distributed from sea level to the Tibetan Plateau under natural conditions. Showing a strong adaptation to the various natural environments, rhesus macaque is considered as an ideal model for exploring adaptive
mechanism of NHPs to natural environments. So far evidence has accumulated that host adaption are associated with gut microbiota which arouse our interest: is the gut microbiota of Rhesus Macaque extend their adaption to various environment especially in extremely environment such as the Tibetan Plateau? However, researches focused on gut microbiotas assisting in high altitude adaptation are limited and mainly conducted on high-altitude herbivores such as pika, yak and tibet sheep. These animal have a large differences with NHPs or rhesus macaques in diet and phylogenetic relationship which may have different adaption mechanism on microbita. Although our preliminary work revealed that significant differences in diversity and the abundance of the core common microbiota between rhesus macaque populations with various altitude gradient, little is known about even the basic feature of high-altitude rhesus macaque microbial community [44].

Here, we collected fecal samples from four high-altitude (above 3000m) wild rhesus macaques populations. Thus we can explore gut microbial community characteristics of high-altitude macaque from multiple populations and get a general conclusion. To more explicitly characterize the gut microbiome in high-altitude populations, samples from one population living in low-altitude environment (below 100m) also collected.

In this study we are trying to answer the question: What is the typical feature of the gut microbiota diversity, composition and function in high altitude populations?

**Fig.1**

**Methods**

**Ethics Statement and Fecal Sample Collection**

Before sample collection, all the animal work was approved by the Institutional Animal Care and Use Committee of the Sichuan Agricultural University (permit number SKY-S20171007). All Rhesus macaques fecal were collected at natural habitats with little disturbance from human and livestock. Samples were collected with sterile gloves
immediately after each wild rhesus macaque had defecated, and were kept cool in a thermos with ice pack. The samples used for bacterial DNA preparation were taken from the inside of the feces under sterile conditions. Then samples were transported to the laboratory on dry ice and stored in -80°C before DNA extraction.

There are five Rhesus macaques populations involved in this study, four groups in high-altitude habitats and one in low-altitude habitats. The low-altitude fecal samples were collected at LingShui (shorted as LS). LS with an average elevation of 100 meters below (in Hainan province) belongs to tropical monsoon climate with a indistinct seasons, high-oxygen and high-temperature and producing a considerable amount of high quality food (especially for mango and coconut in the study site) throughout the year, which is a hospitality environment for Rhesus macaques. All high-altitude habitats at the Tibet Plateau with an altitude above 3000m including JiangDa, BaiYu, YuShu and GongBujiangda shorted as JD, BY, YS and GB respectively. By the way, JD population is relatively closed to BY population (crow-fly distance 70km) but seperated by the Yangtze river. This is for the propose that to investigate if the river could be a potential geographical barrier to gut microbial community of rhesus macaques.

**DNA Extraction and Sequencing**

DNA was extracted from fecal samples using a TIANamp Stool DNA kit (Tiangen, Beijing, China), following the manufacturer's instructions. The integrity of the extracted genomic DNA was verified by 1.0% agarose gel electrophoresis. V3-V4 regions of bacterial 16S rRNA gene (from 341 to 806) were amplifed from extracted DNA using barcoded primers 341 F (5′- CCTACGGGNGGCWGCAG -3′) and 806 R (5′-GGACTACNVGGGTATCTAAT-3′). PCR was performed in a 50 μL reaction system containing 1.5 μL of each primer, 100 ng template DNA, 5 μL10× KOD Buffer, 5 μL 2.5 mM dNTPs and 1 μL KOD polymerase. The
PCR conditions consisted of a denaturation step at 95 °C for 2 min, the amplifications were carried out with 27 cycles at a melting temperature of 98 °C for 10 sec, an annealing temperature of 62 °C for 30 sec, and an extension temperature of 68 °C for 30 sec. Finally, an extra extension step at 68 °C for 10 min was performed. The barcoded PCR products were purified using a DNA gel extraction kit (Axygen, China) and quantified using QuantiFluorTM real-time PCR. Then, next-generation sequencing was performed by Illumina Hiseq 2500 PE250, which was conducted by Genedenovo Inc. (Guangzhou, China).

Data Analyses

Low quality sequences that containing the ambiguous base (N) more than 10% were removed, and sequences with high-quality base (quality score above 20) less than 60% were also removed. Then tags were merged using the FLASH program (version1.2.11) with default parameters. Low-quality contigs were removed using Qiime(V1.9.1)[45]. After removing chimera, the software MOTHUR was used to remove the redundant tags to get unique tags[46, 47]. The obtained unique tags were then used to calculate the abundance. The demultiplexed reads were clustered at 97% sequence identity into operational taxonomic units (OTUs) using the UPARSE pipeline[48]. OTUs were classified using RDP classifier and the Silva version 123 and Greengenes version gg_13_5 reference set with a 80% confidence threshold[49-51]. After annotation, we find three samples (YS-1 YS-2 LS-9) were abnormal higher in Proteobacteria and Actinobacteria which may reflect the dysbiosis in gut microbiota [52]. So we removed these samples in the subsequent analyses.

The alpha diversity index Shannon, Simpson, Chao1, ACE index, and rarefaction curves were calculated using Qiime. The Beta Diversity metrics, the weighted and unweighted UniFrac distance matrices were calculated and visualized with R statistical software.
Statistics of welch’s t-test, wilconxon rank sum test, adonis and anosim test was also calculated using R. The function of gut bacteria communities were predicted using phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt version 1.1.0)[53].

Results

Sequencing Profiles

After quality filtering, 6873729 16S ribosomal RNA gene sequences were obtained from 50 rhesus macaque feacl sample (137474±21752 per sample). Then sequences were clustered at 97% sequence identity and 3005 otus were generated(1234±181 per sample) (Table S1). The rarefaction curves had already reached a plateau at this sequencing depth(Fig.S1), suggesting that the sequencing depth had met the demand for sub-sequence analysis.

Gut microbiotas diversity in high altitude populations

Firstly, we calculated the alpha diversity indexes to evaluate the gut microbiotas diversity in high altitude populations. The Chao1 (1519±147), ACE (1494±147), sob (1158±124), shannon (6.670±0.2280) and simpson(1-D=0.9704±0.005958) index have been profiled. In detail, GB and YS showed a similarly higher Chao1 index are 1659 and 1658; JD was in the middle(1444) and BY group was relatively lower in 1314(Fig.2; specific data of each samples are presented in table S2 ). Specially, the Chao1 index showed significant difference between all high-altitude groups(two-tailed t-test P<0.05) except between GB and YS(two-tailed t-test P=0.98). The results of ACE, sob and shannon indexes were similar to Chao1 indexes. In contrast, the differences in simpson indexes between all groups were more slightly. Simpson indexes were highest in the GB group and only reached significant level with the lowest group(two-tailed t-test P<0.05).
When compared with low-altitude populations, the high-altitude populations exhibited a series feature of high alpha diversity, for example, shannon and simpson indexes are higher in all high-altitude populations, although lack a significant difference when compared with BY and JD separately (comparison of alpha diversity between all groups can be founded in Table S3 ). But if we merged all high-altitude populations as a group and compared with low-altitude population, the difference reached a significant level (Fig.3 two-tailed t-test for shannon p=0.01 and for simpson p=0.005). This result also was validated by Wilcoxon rank sum test even with a stricter confidence level (p=0.007 and p=0.0003 for shannon and simpson respectively). Other alpha diversity indexes such as Chao1 index (Fig.3) also indicated that the high-altitude population with a relatively high alpha diversity.

We measured beta diversity index (bray, jaccard, weighted and unweighted Unifrac distances) to untangle inter-individual variation among all Rhesus macaques gut microbial communities. Based on weighted Unifrac distances, We found inter-individual variation were much higher in high-altitude populations GB and JD and the other two populations YS and BY were relatively lower. And this result was maintained when using bray distances and jaccard distances (Fig.S2). Given that we detected a higher alpha diversity in high-altitude populations, we compared beta diversity between two altitudes and found it also significant higher in high-altitude population based on weighted Unifrac distances (Fig.S2 P<0.05 between all group, except between BaiYu and LingShui P=0.08).
Based on weighted and unweighted Unifrac distances, UPGMA cluster analysis have been conducted to visualize the results of beta diversity. We conduct low-altitude population LS as the outgroup, then the unweighted Unifrac UPGMA clustering tree divided into two clade, I and II. Clade I consisted of high-altitude population BY; other three high-altitude populations were on clade II(Fig.4a). Although the geography distance between BY, JD and YS were all similar and close(about 100km), JD had closer relationship with low-altitude population LS while samples from YS clustered tightly with GB’s which had a farther distance. We also found that all samples were clustered by regional distribution clearly in unweighted Unifrac UPGMA clustering tree, but it can’t distinct low-altitude population from high-altitude population.

However, patterns in the weighted Unifrac UPGMA tree were different. The most obvious difference was all samples were clustered by altitudes(Fig.4b). In the high-altitude clade, some high-altitude samples were deviated from their region and clustered into other population which indicating the pattern that samples distinceted by regional distribution showed a trend of dispersion when counted the sequence abundance.

Next we performed PCoA to directly visualize the relationship of beta diversity distance among four high-altitude populations. In the weighted Unifrac PCoA polt, samples from high-altitude population GB, YS and JD exhibited the closer relationship while BY distributed below the X-axis and seperated with above-mentioned high-altitude samples slightly(Fig.5). On the other hand, high-altitude population mainly concentrated on the left of Y-axis and Seperated from low-altitude population in the X-axis. And Pcoa1 account
main difference for 42.65% while Pcoa2 account for 14.14%. Suggesting the gut microbial community structure of high-altitude populations differed from low-altitude's. To further validate these differences, we conducted a analysis of similarity (ANOSIM) test on weighted Unifrac and unweighted Unifrac distance results. The result of ANOSIM test proved that there were significant difference between low-altitude and high-altitude populations (unweighted Unifrac distance $r = 0.4895$, $P < 0.001$; weighted Unifrac distance $r = 0.6406$, $P < 0.001$). We also performed a permutational multivariate analysis of variance (PERMANOVA) on weighted Unifrac and unweighted Unifrac distance results. The PERMANOVA results coincided with the ANOSIM (unweighted Unifrac distance $r^2 = 0.1143$, $P = 0.001$; weighted Unifrac distance $r^2 = 0.2955$, $P = 0.001$).

**Fig. 5** PCOA plot based on unweighted Unifrac distances (a) and weighted Unifrac distances (b).

In short, these data suggested that the gut microbiotas of high-altitude population exhibited a feature of relatively high alpha diversity and beta diversity. And it clearly distinkted from low-altitude population in beta diversity distances when the information of abundance was included (weighted).

**Microbial community structure in high-altitude populations**

**Fig. 6**

To figure out the microbita component feature in high-altitude populations, we conducted analysis on the OTU and gut microbiota composition. In this study, sequences more than 99.95% has been annotated into phylum level, the predominant Phylum of high-altitude rhesus monkey populations were Firmicutes (63.16±14.71%) and Bacteroidetes (Fig. 6 a; 24.25±12.07%). Firmicutes were highest in GB group accounting total sequecnes for
75.45%; this number were little lower in YS group(72.59%); and for BY were 55.45%; and even in the lowest group JD(53.05%) this number were significant higher than low-altitude group (35.98% T-test P<0.001). There were a negative correlation between Firmicutes and Bacteroidetes, which means the proportion of Bacteroidetes in GB(16.95%) were less than other high-altitude populations such as BY(20.12%) and YS(22.82%). Notably, the Bacteroidetes of JD were much more higher than other high-altitude groups but still lower than low-altitude group. In general, general Firmicutes and Bacteroidetes constituted about 90%. The subordinate phylum were Spirochaetae(5.29±7.40%), Verrucomicrobia(2.16±2.90%), Proteobacteria(1.82±1.68%), and Actinobacteria(1.43±1.46%); sequences from other phylums were lower than 1%. Notabley, compared with other groups, BY was enriched in Spirochaetae(15.10%). In family level, the rate of annotation ranged from 90.5% to 97.9% (on average 95%), Ruminococcaceae(32.24±8.68%), Lachnospiraceae (12.75±7.32%) and Prevotellaceae(10.46±9.74%) were major family, plused with other represented family such as Bacteroidales_S24-7_group(7.37±6.60%), Spirochaetaceae(5.25±7.42%), Christensenellaceae, Rikenellaceae and Veillonellaceae they contributed sequences more than 80%(Fig.6 b). Particularly, the abundance of Prevotellaceae were highest in JD while Ruminococcaceae were lowest. Specially, when compared with low-altitude group, high-altitude populations contained more Firmicutes(two tailed t-test P<10^{-9}) but less Bacteroidetes(two tailed t-test P<10^{-5}) than low-altitude population. Thus the ratio of Firmicutes to Bacteroidetes in high-altitude populations was more than three times as large as low-altitude population.

To further explore the distinguishing feature in species composition in high altitude populations, t-test was used in family level. There were some significant differences in the
abundances of the gut bacterial communities between samples from different altitude. Christensenellaceae (two tailed t-test P<0.01) and Ruminococcaceae (two tailed t-test P<0.01) were significant high in high-altitude population, and Prevotellaceae was significant high in low-altitude population (fig.7). In addition, Ruminococcaceae and Prevotellaceae were the largest family to high-altitude and low-altitude population, respectively. It is also worth noting that these discrepancy has been detected in all four high-altitude populations when compared with low-altitude population separately.

**Fig.7**

Based on OTUs data, we conducted a venn diagram to explore the common OTUs between populations. To improve the reliability of our data and reflect the gut microbiota community of each group we filtered OTUs which appeared in less than two individual at the same group, and 1642 OTUs were remained. Furthermore, 458 OTUs were shared by all five populations commonly, namely they were rhesus macaques core OTUs, when calculated by abundance they constituted total sequence for 90% (Fig. S3 and Table S4). Besides core OTUs, we found there were 82 OTUs present in all four high-altitude populations constituting total sequences for 2%. Most of these (65 of 82) OTUs were belonged to Firmicutes at the phylum, and most microbes in this phylum belong to the Ruminococcaceae (40 of 82) at family level. This result suggested that differences in unique OTUs might be a small part in abundance, but it was highly consistent in all high-altitude population indicate the important in environment adaption.

**Functional profiling of microbiotas in high altitude populations.**

**Fig.8**

To evaluate metabolic function differences between gut microbiotas communities associated with high altitude, we used Phylogenetic Investigation of Communities by
Reconstruction of Unobserved States (PICRUSt) to infer microbial community function.

Microbial community function has been assigned to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway including 35 level 2 and 221 level 3 ortholog groups. NSTI number used to estimate reliability of this method are at a acceptable value (Fig S4). The gene copy number were highest in the high-altitude population GB(65445) and YS(65163), and BY(57831) and JD(57145) were at a lower copy number. And which was significant higher than low-altitude group(52049) (P=0.0005). In order to verify if this high gene copy is affected by sequence abundance, we rarefied sequence into same reads(30000), and got a similar result. Thus, there were pervasive differences pathway between two altitude populations including 29 level 2 and 160 level 3 ortholog groups (t-test P<0.05). Strikingly, almost all differences pathway were higher in high-altitude population. Functions enriched in high-altitude populations were involved in a wide range of processes, for instance, Membrane Transport, Carbohydrate Metabolism, Amino Acid Metabolism, Replication and Repair, Translation, Energy Metabolism and so on (Fig.8). Specially, only 3 of 29 differences pathway in level 2 were higher in low-altitude. They were Glycan Biosynthesis and Metabolism and Digestive System and Immune System Diseases, but only Glycan Biosynthesis and Metabolism reached a worthy of notice level in abundance(3%), others were in trace abundance (0.04%-0.1%). We changed the species annotation database from SILVA to greengene, replaced t-test with wilcoxon rank sum test to further confirm this phenomenon and came up with a very similar result.

In conclusion, these data indicate that pervasive biological processes were elevated in intestinal microbes of high-altitude Rhesus Macaques when compared with low-altitude population, and mainly contributed by abundance difference in common OTUs.

Discussion

Gut microbial community of high-altitude Rhesus macaques are dominated by Firmicutes
and Bacteroidetes which is similar to low-altitude population. However, in high-altitude populations the ratio of Firmicutes/Bacteroidetes are higher than low-altitude group. In detail, the ratio of Firmicutes/Bacteroidetes in four high-altitude populations are 2.8, 1.4, 4.45, 3.2, while in low-altitude is 0.69, and it differs significantly between altitudes (p<0.001). These results are coincident with research conducted on high altitude Tibetan[54]. Firmicutes are considered to be encode energy metabolism-related enzymes, and can produce many digestive enzymes to decompose various substances while Bacteroidetes are correlated to degrade carbohydrates and proteins[55]. Similar results that increased F/B ratio also observed in cold exposed mouse which was associated with increased non-shivering thermogenesis and energy harvest indicating high-altitude population with high energy harvest and consumption[56].

The high F/B ratio are mainly contributed by the increasement of family Ruminococcaceae (belong to Firmicutes) and the decreasement of family Prevotellaceae (belong to Bacteroidetes). Ruminococcaceae is enriched in high altitude populations while Prevotellaceae enriched in low altitude population. It is known that Ruminococcaceae is enriched genes for cellulose degradation[57]. By microbial fermentation, cellulose can be transform into short-chain fatty acids (SCFA) which are important energy source for epithelium and can provide about 10% energy for human[58, 59]. In black howler monkeys, Ruminococcaceae increased during the energy scarce period and appears to compensate for reduced energy intake. In high-altitude habitat, hypoxia and cold environment requires more energy to maintain metabolic balance, but available food source are limited and (grassroot and bark) mainly consisted of lignin and cellulose which can’t be utililized by host[26]. So, increased abundance in Ruminococcaceae led to increased efficiency in energy intake and help rhesus macaques’ living in cold high-altitude environment. Prevotella can utilize carbohydrate
efficiently[60]. It has also been validated that Prevotellaceae have a positive correlation with fruit consumption in NHPs like western lowland gorillas and Verreaux’s sifakas[59, 61]. And this result is coincident with our observation that high altitude habitat belonging to plateau climate with a limited fruit production. However, a research focused on high-altitude ruminants found Prevotella is higher than their relatives in low-altitude[41]. But there are also differences in phylogenetic relationship, physiology and food habit between ruminants and NHPs which are all influence factors to gut microbial community[62]. This discrepancy implies various species may have diverse changes in gut microbota composition to high-altitude environment adaptation.

In addition, our previous work based on elevational gradient found abundance of Christensenellaceae was significantly higher in Tibet (3427m) than in other geographical populations (5m, 158m, 1161m, 1629m, 2895m)[44]. But there are still some shortcomings that only one high-altitude site with six samples were included which may not represent the universal feature of microbiotas composition in high-altitude environment. In this study, we find the abundance of Christensenellaceae in high-altitude populations BY, JD, GB and YS are 2.0%, 2.2%, 6.4% and 2.6%, respectively. However, the abundance of Christensenellaceae in low-altitude population LS are only up to 0.77% which are significantly lower when compared to all high-altitude populations(p<0.01). Therefore, we could infer that Christensenellaceae are pervasive higher in high-altitude rhesus macaques populations and may play a critical role in high-altitude adaptation.

Previous research found Christensenellaceae is significantly correlated with lean body shape (body mass index (BMI)<25), and the abundances of Christensenellaceae were highly associated with lower triglyceride[19, 63, 64]. But these research didn’t illustrate the specific mechanism that how Christensenellaceae interact with host Physiology and how Christensenellaceae reduce weight and total adiposity gain. In our study, high-
altitude Rhesus Macaque are living a cold environment with high energy requirement which is proved by increased in abundance of Firmicutes. Therefore, Christensenellaceae are less likely to reduce weight by suppressing energy harvest. We speculate the mechanism might be that Christensenellaceae can stimulate host metabolic rate and increase energy consumption. This high metabolic rate could help Rhesus Macaque maintain their body temperature during the cold winter. This hypothesis is partial implied by other study that find the relative abundance of Christensenellaceae has a markedly positive correlation with food intake, body mass change and athletic ability[65].

Specially, we find every population contains Treponema (belong to Spirochaetae) at the proportion of 1%-3% except BY group which contains Treponema up to 15%. Usually, Treponema is known for T. pallidum, the cause of syphilis and yaws. This genus also includes proficient cellulose and xylan hydrolyzers, and it is pervasive in NHPs but rare in industrialized human populations[66, 67]. In a study focused on western lowland gorillas, Treponema was the only genus associated with low fruit yield[61]. Treponema could be an adaptation to diets riched in fiber, and it is possible that Treponema help the host to extract nutrients from the fibrous foods by degradating fibre. High Treponema content in BY group may imply less desirable food and high fibre intake, but the specific factor needs more investigation on surrounding areas.

In contrast to widespread in NHPs, Treponema is found only in a few rural communities with a substantial content, like Hadza whose diet is high in fiber riched plant foods[67]. Fibre degraders can help release nutrition from fibre riched foods. On the other hand, Fibre riched food could promote the growth of these species. Western diet that consisted of high fat and low cellulose may lead to the exhaustion of fibre degraders which plays an important role in evolutionary history. Study focused on gut microbiota of wild NHPs may provide a excellent view on microbiome-associated human disease.
High-altitude diversity

In the Venn diagram, all rhesus macaques groups share 458 otus (core otus) and they constitute most of microbiome of an individual which means they may be critical important to the essential function of rhesus macaques. But we also found 82 OTUs shared by four high-altitude populations with far geographical distance. Although these high-altitude OTUs are less in number, the consistency in all high-altitude populations indicate they may play an important role in high-altitude environment adaption. We also observed that high-altitude populations exhibit a feature of high alpha diversity in multivariate alpha index, and this situation is similar to high-altitude pika[40]. Evidence has accumulated that the gut microbiome harbors 130 glycoside hydrolase, 22 polysaccharidelyase, and 16 carbohydrate esterase families[57]. High alpha diversity are equal to more microbial species which contains more cellulose-associated gene. Diversity of genes provide diverse parallel pathways, which provide the microbiome high flexibility and high capacity to utilize different energy sources of cellulose[40].

High-altitude group BY and JD exhibit relatively lower alpha diversity than other high-altitude populations. Interestingly, both groups are very close to the Yangtse River in geography. We may suspect the Yangtse River provide higher environment humidity resulting a relative luxuriant vegetation. Thus, rhesus macaques in this environment may have more available food resource and don’t rely as heavy as other high-altitude population on diversity microbita to assimilate indigestive food.

On the other hand, a recent research revealed that our body have the ability to control microbial communities by restricting nitrogen access to starve gut bacteria or secreting nitrogen via the intestinal cells to feed the bacterias[68]. Compared to low-altitude populations, high-altitude population consume less high-nitrogen levels food, for example fruit, which might weaken the control from host. In our data, otus in abundance rank top
five for low-altitude are 17421, 9709, 5407, 3870, 3177, respectively. In high-altitude they are 6025, 4621, 4244, 3203, 3191. So, some bacterias in low-altitude population might multiply excessively and finally lead to both low OTUs and low evenness which is the premonition of imbalance. Similar phenomenon are also observed in human who have a west diet which constituted of massive fat and protein[69].

Gut microbiota composition are shaped by many factors such as genetic background, diet, environmental differences, and social behavior[20, 25, 70, 71]. In the same population, animals are similar in genetic background and diet share and interact with the same environment, it’s expectable that gut microbiota composition of Rhesus Macaque are more similar in the same group than different group. The result of unweighted Unifrac UPGMA cluster dendrogram is at expectation that samples are clustered as geographical populations, and also indicate river has a stronger isolation effectiveness than mountain to gut microbiota community of rhesus macaques. However high-altitude populations can’t be distinct from low-altitude population outgroup in this dendrogram. Interestingly, in the weighted dendrogram high-altitude populations are separated from low-altitude population, but individual doesn’t clustered as geographical populations strictly. These data suggest that high-altitude environment may shape gut microbiota composition in a similar way which beyond differences in genetic background, geographical position, and social relationships.

**Pervasive enrichment of gut microbiota function in high-altitude Rhesus Macaque**

All mammals have a symbiotic relationship with their microbial community which contains $10^{13}$ bacterial cells and more than 3 million genes which enable the community to carry out multiple function[72, 73]. Unlike the host genome, the microbiome can change the composition of the microbial community or evolve rapidly in individual microbial genes,
resulting in modified transcriptomic, proteomic and metabolic profiles[74, 75]. Recent research has revealed that gut microbiota of NHPs, vary from season[26, 27]. The dynamic fluctuations in the microbiota community that are associated with tremendous changes in gene copy number and appears to help host adaption to their environment. In this study, we exert PICRUSt to infer the function of Rhesus Macaque gut microbial community. We supposed that genes involved in metabolism may enriched in high-altitude population. Spectacularly, almost all gene copy number are higher in all high-altitude populations than low-altitude population. In level two, top five pathway in both altitude are Membrane Transport, Carbohydrate Metabolism, Amino Acid Metabolism, Translation, Replication and Repair. And these mentioned pathway are significant higher in high-altitude. ATP-binding cassette (ABC) transporters ,the highest pathway in Membrane Transport catalogue, is involved in ATP generation directly. Genes enriched in Carbohydrate Metabolism, Translation and Amino Acid Metabolism suggest high metabolism capacity of gut microbial community. Low oxygen and high ultraviolet radiation may cause DNA and protein impairment in high-altitude environment, genes related to Replication and Repair are conducive to reduce the damage of biological molecules. Thus, these pathway may help Rhesus Macaque adapt high-altitude environment. In abundance ranking top 20, only Glycan Biosynthesis and Metabolism pathway is significant higher in low-altitude population and are concentrated in Lipopolysaccharide biosynthesis. Lipopolysaccharide is the major component of the outer membrane of Gram-negative bacteria and also a typical endotoxin[76]. It’s in coincidence with our hypothesis that excessive nutrition in low-altitude population result in reproduction of specific bacteria which is the premonition of disorder.

Conclusions

We found high-altitude rhesus macaques possesses a distinct gut microbial community
when compared with low-altitude population. And these changes in rhesus macaques gut microbiota were mainly caused by their high-altitude habitat environment which contained the feature of high-fiber diet, cold temperature and hypoxia.

Abbreviations

NHPs: non-human primate; OTU: operational taxonomic unit; 16S rRNA: 16S ribosomal RNA; SCFAs: short-chain fatty acids; Chao1: The Chao1 estimator; ACE: Abundance-based coverage estimator; UPGMA: unweighted pair-group method with arithmetic means; ANOSIM: analysis of similarity; PERMANOVA: permutational multivariate analysis of variance; PICRUSt: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; KEGG: Kyoto Encyclopedia of Genes and Genomes; NSTI: Nearest sequenced taxon index;

Declarations

Ethics approval and consent to participate

All the animal work was approved by The Institutional Animal Care and Use Committee of the Sichuan Agricultural University (permit number SKY-S20171007)

Consent to publish

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests
The authors report no conflict of interest.

Authors’ contributions
Huailiang Xu, Yuhan Wu and Yongfang Yao designed the experiment and wrote the first draft. Mengmeng Dong and Tianrui Xia collected the fecal samples and performed preliminary preparation. All authors has helped in revision and approved the final manuscript.

Funding
This work was supported by the National Natural Science Foundation of China under Grant (31870355). The funding bodies had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Acknowledgements
We would like to extend our sincere gratitude to JunSong Zhao, Pu Zhao, Qian Su and Xue Liu for their assistance in sample collection.

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Figures
Sample collection map. The base county-level vector map was downloaded from National Geomatics Center, and the collection sites are inserted by longitudinal and latitudinal coordinate. Rivers are showed in blue lines and sample collection sites are marked with square with number. 1-GongBujiangda about 3200m 2-YuShu 4080m 3-JiangDa 3246m 4-BaiYu 4091m (low-altitude group LingShui below 50m is limited). * The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries.

This map has been provided by the authors.
Profile of alpha diversity by (a) chao1 index, (b) shannon and (c) simpson index.

The difference of gut microbiota alpha diversity between high-altitude and low-altitude population. (a) chao1 index (b) shannon index (c) simpson index. The significance is indicated by *P<0.05, **P<0.01, ***P<0.001
UPGMA cluster plot based on unweighted Unifrac distances (a) and weighted Unifrac distances (b).

Figure 4
Figure 5
PCOA plot based on unweighted Unifrac distances(a) and weighted Unifrac distances(b).

Figure 6
The compositions of high-altitude rhesus macaques gut microbiota at phylum level and family level. Abundance out of top 10 are be classified as other in this plot.
Figure 7

Compositions feature of high-altitude population. The significance is indicated by

\[ *P<0.05, \quad **P<0.01, \quad ***P<0.001 \]
The comparison of gene copy number between high-altitude and low-altitude population. The significance is indicated by *P<0.05, **P<0.01, ***P<0.001

**Supplementary Files**

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