A preliminary study of the salivary microbiota in young male subjects under acute high-altitude exposure

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Background. The microbial community structure in saliva differs in residents at different altitudes. However, the impact of acute exposure to high altitude on the oral microbiota is unclear. This study explored the impact of acute altitude exposure on the salivary microbiome to establish a foundation for the prevention of oral diseases.

Methods. Unstimulated whole saliva samples were collected from 12 male subjects at the following three time points: one day before entering high altitude (an altitude of 350 metres, pre-altitude group), seven days after arrival at high altitude (an altitude of 4500 metres, altitude group) and seven days after returning to low altitude (an altitude of 350 metres, post-altitude group). Thus, a total of 36 saliva samples were obtained. 16S rRNA V3-V4 region amplicon sequencing was used to analyze the diversity and structure of the salivary microbial communities, and network analysis was employed to investigate the relationships among salivary microorganisms. The function of these microorganisms was predicted with a Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis.

Results. In total, there were 756 Operational Taxonomic Units (OTUs), and we identified 541, 613, and 615 OTUs in the pre-altitude, altitude, and post-altitude group. Acute altitude exposure decreased the diversity of the salivary microbiome. Prior to acute altitude exposure, the microbiome mainly consisted of Proteobacteria, Firmicutes, Bacteroidetes, Fusobacteria, and Actinobacteria. After altitude exposure, the relative abundance of *Streptococcus* and *Veillonella* increased, and the relative abundance of *Prevotella*, *Porphyromonas*, and *Alloprevotella* decreased. The relationship among the salivary microorganisms was affected by acute altitude exposure. The relative abundance of carbohydrate metabolism gene functions was upregulated, while that of coenzyme and vitamin metabolism gene functions was downregulated.

Conclusion. Rapid high altitude exposure decreased the biodiversity of the salivary microbiome, changing the community structure, symbiotic relationships among species, and abundance of functional genes. This suggests that the stress of acute altitude exposure influenced the stability of the salivary microbiome.

Keywords Acute altitude exposure, Saliva, 16S rRNA, Microbiota, Diversity

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Abstract

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Introduction

In recent years, the number of people travelling to high altitudes has increased due to the availability of transportation and travel for work, sports events, and even earthquake relief (Seccombe and Peters, 2014; Hodkinson, 2011). In some cases, travellers may experience acute exposure to high altitudes, which occurs when individuals ascend to areas over 2,500 meters above sea level within 24 hours. High-altitude environments have low atmospheric pressure, low oxygen levels, large temperature differences, and strong ultraviolet radiation (Lackermair et al., 2019; Torlasco et al., 2020; Swenson et al., 2020). Therefore, acute exposure to high-altitude environments can have negative health consequences, such as increased blood pressure and increased platelet aggregation, and may even result in acute high-altitude sickness (Revera et al., 2017; Lackermair et al., 2019; Luks et al., 2017). Evidence has indicated that acute exposure to high altitude may also induce oral diseases. For example, patients with no previous history of toothache developed symptoms of acute pulpitis within 48 hours of flying to high-altitude locations; this onset is thought to be related to high-altitude hypoxia, decreased atmospheric pressure, and external stimulation of dental pulp after dental caries (Zhang et al., 2020). Periodontitis, gingivitis, and oral ulcers are the most common oral diseases upon travel to high altitudes (Li and Liu, 2018). Additionally, a rat model developed more serious periodontal damage under simulated high-altitude hypoxic conditions (Xiao et al., 2012). Moreover, while oral diseases can result in direct issues and symptoms, they also indirectly increase the risk of systemic diseases such as myocardial infarction, infective endocarditis, and respiratory diseases to a certain extent (Sampaio-Maia et al., 2016).

The aetiology of oral diseases induced by high-altitude environments is complex. Microorganisms are the initiating factor of oral infection and thus are closely related to the occurrence of disease (Arweiler Nicole B, 2016). In healthy individuals, the oral microbial community maintains a dynamic balance, which provides protection against adverse external stimulation. An imbalance in microorganisms or between microorganisms and the host increases the risk of dental caries and periodontitis (Zhang et al., 2018). Among them, the microorganisms in oral plaque biofilm are considered to be the pathogenic bacteria in the development of periodontal disease. These pathogenic bacteria include the red complex bacteria (*Tannerella forsythia*, *Treponema denticola* and *Porphyromonas gingivalis*), which drive the pathogenesis of periodontal disease by regulating the recombination of microbiota and promoting inflammatory response (Valm, 2019). In addition, Studies have found that oral microbiota has been associated with the development of oral cancer and primary tumors beyond the head and neck (Mascitti et al., 2019; Zhang et al., 2018 Stasiewicz et al., 2022; Liu et al., 2022). Epidemiological studies have shown that periodontal disease is associated with an increased risk of esophageal, gastric, pancreatic, and colorectal cancers (Michaud et al., 2017; Hu et al., 2018; Fan et al., 2018). This may be related to dysbiosis of the oral microbiota leading to the development of chronic inflammation, altered metabolic activity leading to increased production of toxic metabolites and

immune responses that promote tumorigenesis and tumor growth (Snider et al., 2016; Inaba et al., 2014; Gainza-Cirauqui et al., 2013).

Altitude, atmospheric oxygen levels, temperature, psychological stress, disturbances in circadian rhythms, and sleep deprivation all affect the composition, distribution, and metabolic activity of oral microorganisms (Gao et al., 2018; Lamont et al., 2018; Liu et al., 2021). The oral cavity has multiple unique niches and a symbiotic bacterial ecosystem. The five major niches include the saliva, tongue surface, oral mucosa, tooth surface, and subgingival plaque. Each niche has a different microbial community (Consortium, 2012). Saliva contains more than 700 different microbial species, which makes saliva a potential pool of biomarkers (Wang et al., 2022). In orthodontic patients, the diversity of the salivary microbiome was lower at high altitudes; the relative abundance of *Streptococcus* in the salivary microbiome increased, while the relative abundance of *Veillonella* decreased (AlShahrani et al., 2020). These studies suggest that the salivary microbial community differs between high- and low-altitude residents, but currently, no studies have investigated whether acute altitude exposure influences the composition and functional metabolism of the salivary microbial community.

Therefore, the aim of this study was to examine changes in the composition and structure of the salivary microbial community after acute high-altitude exposure and to explore the correlation between bacteria and bacterial functions. The results of this study expand the current understanding of the influence of high-altitude exposure on salivary microorganisms, providing a theoretical foundation for the future development of preventive measures for acute high-altitude oral diseases.

Materials & Methods

Study Subjects

The study protocol was approved by the ethics review board of PLA Strategic Support Force Medical Center (No. K2021-10). We have obtained written informed consent from all study participants. All of the procedures were performed in accordance with the Declaration of Helsinki and relevant policies in China.

The subjects were recruited from a scientific research unit in a city in northern China. The inclusion criteria for the subjects were as follows: (1) ethnic Han male, over 18 years old; (2) long-term resident of the low-altitude plain; (3) no oral infectious diseases; (4) no systemic or genetic disease; and (5) no history of smoking. The exclusion criteria were as follows: (1) subjects who had travelled to high-altitude locations within the past six months; 2) subjects who could not tolerate a high-altitude environment; (3) subjects who had received antibiotics, hormones, or other drugs within the past 3 months; and (4) subjects who had received periodontal treatment within the past 3 months. Finally, 12 healthy male subjects aged from 26 to 45 years (mean age 34.9±7.0 years) were recruited in this study, and all subjects agreed to participate.

Sample collection

This study examined the salivary microbiome of 12 subjects who were transferred from a plain (at an altitude of 350 meters) to a plateau (at an altitude of 4500 meters) within a period of 12 hours. All subjects lived in a hotel and stayed at high altitude for 7 days. Their physical activity was walking during their stay at the plateau. The intensity and duration of their physical activity were similar. Over the course of the travel, unstimulated whole saliva samples were collected from each subject at three different time points: one day before reaching the plateau (pre-altitude group), the 7th day after entering the plateau (altitude group) and the 7th day after returning to the plain (post-altitude group). Thus, a total of 36 saliva samples were obtained.

All participants were required to fill in a unified oral health questionnaire before and after entering the plateau. In addition, in order to minimize the impact of other interference factors on saliva samples, the following requirements were made for the subjects during the study: (1) the diet of each subject was uniformly provided by the study organization. (2) Before the start of the study, a professional stomatologist taught all subjects about the brushing method (pasteurization brushing method), and tried to ensure that the brushing method and time were the same. (3) Use a uniform toothbrush and toothpaste. Samples were collected from 9 a.m. to 11 a.m., which is consistent with previous studies (Gill et al., 2016). During collection of the samples, the subjects first rinsed their mouth with deionized water and then were instructed to sit quietly for 10 minutes. Then, a disposable sterile saliva collection tube was placed on the oral mucosa of the subject's lower lip, allowing saliva to naturally flow into the collector. After approximately 2 ml of saliva had been collected, preservation solution was added to the collection tube, and the sample was stored in a -20°C refrigerator, and then transferred to plain areas through cold chain transport (liquid nitrogen) to be stored at -20°C for further study (Gill et al., 2016).

Bacterial DNA extraction and PCR

Total genomic DNA from the samples was extracted using the cetyltrimethylammonium bromide (CTAB) method (Kachiprath et al., 2018). DNA concentration and purity were assessed on 1% agarose gels (Beijing, China). Then, the DNA concentration was diluted to 1 ng/μL with sterile water. Specific barcode primers were used to amplify the DNA in the 16S V3-V4 sequencing region with high-efficiency, high-fidelity enzymes (Phusion® High-Fidelity PCR Master Mix, New England Biolabs). All PCR products were run on 2% agarose gels for detection, and the PCR products were purified with a Qiagen Gel Extraction Kit (Qiagen, Germany).

DNA library preparation and Sequencing

Sequencing libraries were generated using the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following the manufacturer's recommendations; index codes were also added (Yang et al., 2019). FLASH (V1.2.7 http://ccb.jhu.edu/software/FLASH/) was used to splice reads from each sample to obtain raw tags, which were rigorously filtered to obtain high-quality tags (Magoc and Salzberg, 2011) using the QIIME

(V1.9.1 http://qiime.org/scripts/split_libraries_fastq.html) operation procedure (the default quality threshold was <=19) (Bokulich et al., 2013). The resulting library was sequenced on an Illumina NovaSeq platform, and 250 bp paired-end reads were generated.

Data analysis

All effective tags were clustered into operational taxonomic units (OTUs) by using UPARSE software (V7.0.1001 http://drive5.com/uparse/, 97% identity) (Sevinsky et al.,2010). Through OTU annotation analysis, taxonomic information and the community composition were obtained (Haas et al., 2011; Edgar, 2013). A Venn diagram was used to define the shared and unique microbiome at the species level by OTU clustering. Alpha and Beta diversity was analysed to investigate differences in microbial community structure among groups (Simons et al., 2019). Additionally, principal coordinate analysis (PCoA) was performed to obtain principal coordinates and visualize complex, multidimensional data (Wang et al., 2020). Analysis of similarity (ANOSIM) was used to determine differences in community structure among groups and to compare the differences within and between groups (Yang et al., 2019). Network analysis was used to determine the relationships among dominant genera by calculating the correlation coefficient (Niquil et al., 2020). Based on the abundance of bacteria, PICRUSt analysis was used for functional prediction of microbial communities in the saliva samples (Douglas et al., 2018).

Statistical analysis

To evaluate the differences in Alpha diversity, taxa at levels, metabolism, and Beta diversity, the normality test was first examined by the Shapiro-Wilk test, and *P* values were reported. The Alpha diversity, taxa at levels, metabolism, and Beta diversity were evaluated by using Student's t-tests if $P < 0.05$, else were evaluated by using non-parametric (Wilcoxon) tests. as in previous studies. *P* values < 0.05 were considered significant. Alpha diversity, taxa at levels, and metabolism were evaluated using Student's t-tests. Beta diversity was evaluated using non-parametric (Wilcoxon) tests for significance test. The statistical analysis was performed in SPSS 24.0.

Results

Basic information of study subjects

After entering the plateau, 3 subjects showed symptoms of gingival swelling and pain, and 2 patients showed symptoms of oral ulcer. None of the 12 subjects showed positive signs after returning to the plain (Table S1).

Global sequencing data

In total, 3,138,051 raw sequences were generated from the 36 saliva samples, with an average of 87,168 raw sequences per sample (Table S2). Quality filtering was used to acquire 2,298,989 effective sequences, with an average of 63,861 sequences per sample. The shortest sequence

length of the effective tags was 418 bp, the longest sequence length was 425 bp, and the average sequence length was 422 bp (Table 1).

Clustering of all effective sequences was based on a threshold of 97% identity. These sequences were then annotated against the Silva138 database to determine species taxonomy. In total, there were 756 OTUs, of which 653 could be annotated with the database. The number of annotated OTUs at the phylum level was 625, and that at the genus level was 520.

In the Venn diagram, we identified 541, 613, and 615 OTUs in the pre-altitude, altitude, and post-altitude groups, respectively. These groups shared a total of 466 OTUs. A total of 26, 83, and 80 unique OTUs were found in the pre-altitude, altitude, and post-altitude groups, respectively (Fig.1A).

Bacterial diversity analysis

The species accumulation curves tended towards saturation, indicating that the amount of sample was sufficient (Fig.1B). The ACE and Chao1 richness indexes were higher in the post-altitude group than in the pre-altitude and altitude groups, but there were no significant differences among the groups. The Shannon and inverse Simpson diversity indexes were significantly lower in the altitude group than in the pre-altitude and post-altitude groups. Simpson's evenness index was higher in the pre-altitude group than in the altitude group, indicating that the bacterial community distribution in the salivary samples was very uneven. In addition, Good's coverage index was 99.9% for each group, indicating that the sequencing depth was sufficient to detect the bacterial diversity of the saliva samples (Table 2).

Based on the weighted UniFrac distances, the PCoA of beta diversity showed that the samples formed well-separated clusters corresponding to the three groups, suggesting that the oral microbiota community structure differed among the three groups (Fig.1C). The ANOSIM based on the Bray–Curtis distances of the salivary microbiota structure revealed significant differences between the pre-altitude and altitude groups (Fig.1D), altitude and post-altitude groups (Fig.1E), and pre-altitude and post-altitude groups (Fig.1F). Thus, differences in oral microbiota community structure between each group were observed.

Bacterial abundance and distribution

The predominant bacteria were largely consistent among the three groups, but differences in relative abundances were observed. The ten predominant phyla included Proteobacteria, Firmicutes, Bacteroidetes, Fusobacteria, Actinobacteria, Spirochaetes, Tenericutes, unidentified Bacteria, Cyanobacteria, and Synergistetes (Fig. 2A). Among the predominant phyla with a mean relative abundance > 1% in each group, the relative abundance of Firmicutes was significantly higher and that of Bacteroidetes was significantly lower in the altitude group than in the pre-altitude group, and the relative abundance of Actinobacteria was significantly higher and that of Bacteroidetes was significantly lower in the post-altitude group than in the pre-altitude group. Compared to that in the altitude group, the relative abundance of Firmicutes was significantly lower in the post-altitude group (Fig. 2B, C and D).

The ten predominant genera included *Streptococcus*, *Neisseria*, *Haemophilus*, *unidentified Prevotellaceae*, *unidentified Rhizobiaceae*, *Citrobacter*, *Veillonella*, *Fusobacterium*, *Leptotrichia*, and *Prevotella* (Fig.2E). Among the predominant genera with a mean relative abundance > 1% in each group, significantly higher relative abundances of *Streptococcus* and *Veillonella* were observed in the altitude group than in the pre-altitude group, whereas significantly lower relative abundances of *unidentified Prevotellaceae*, *Prevotella*, *Porphyromonas* and *Alloprevotella* were observed in the altitude group than in the pre-altitude group. Additionally, compared to those in the pre-altitude group, significantly lower relative abundances of *Haemophilus*, *unidentified Prevotellaceae*, and *Porphyromonas* were observed in the post-altitude group. Compared to those in the altitude group, the relative abundances of *Streptococcus*, *Haemophilus* and *Veillonella* were significantly lower in the post-altitude group, while the relative abundance of *unidentified Rhizobiaceae* was significantly higher in the post-altitude group (Fig. 2F, G and H). These genera changed in more than half of the subjects. In addition, we found that three subjects developed gingival swelling and pain symptoms and two subjects developed oral ulcer symptoms after entering the plateau. However, no significant difference was found between the positive and asymptomatic subjects in the changes of oral dominant bacteria (Fig. S1).

Network analysis

Network analysis was used to explore the bacterial co-occurrence patterns and provide insight into interactions among the salivary microbiota. There were 736 edges and 91 nodes in the pre-altitude group, 601 edges and 89 nodes in the altitude group. The pre-altitude group had an average path length (APL) of 3.363, a network diameter (ND) of 9 and a graph density (GD) of 0.074. The structural properties of the pre-altitude group network differed from those of the altitude group network (Table S3).

The network diagram for the 100 predominant bacteria at the genus level were shown in pre-altitude and altitude groups (Fig. S2, Fig. S3). There was a higher relative abundance of *Streptococcus* in the pre-altitude group and altitude group. The *Streptococcus* were strongly correlated with *Veillonella*, *Prevotella* and *Fusobacterium* in the pre-plateau group, respectively. However, The Streptococcus were strongly correlated with *Neisseria* and *Haemophilus* in the altitude group, respectively.

PICRUSt function predictions

PICRUSt analysis was performed to predict the potential functions of the salivary microbiota. The ten predominant gene functional pathways were predicted in Kyoto Encyclopedia of Genes and Genomes (KEGG) level 1, the most abundant of the gene functional pathways was metabolism (Fig. 3A). The ten predominant gene functional pathways predicted in KEGG level 2 (Fig. 3B).

The pre-altitude and altitude groups had different KEGG profiles in hierarchy level 3. Ten pathways were significantly different between the pre-altitude group and the altitude group.

Notably, the relative abundance of the carbohydrate metabolism gene functions was upregulated in the altitude group, and the metabolism gene functions of cofactors and vitamin was downregulated in the altitude group (Fig. 3C). These results indicate that the gene function of the salivary microbiota changed upon acute altitude exposure.

Discussion

Rapidly ascending to a high altitude has been shown to have negative effects on health and may contribute to the development of oral diseases. However, while the salivary microbiome of residents at high altitudes is known to differ from that of residents at low altitudes, the effects of acute altitude exposure on the salivary microbiome remain unclear. This study showed that acute altitude exposure decreased the diversity of the salivary microbiome and influenced the relationships among salivary microorganisms. The function of the microbiome was also altered by high-altitude exposure: genes involved in carbohydrate metabolism were upregulated, while genes involved in coenzyme and vitamin metabolism were downregulated. These results provide insight into the development of oral diseases at high altitudes.

The relationship between the salivary microbiome and oral and systemic health has been widely studied (Duran-Pinedo, 2021; Sampaio-Maia et al., 2016). Previous studies have found that participants experienced symptoms of oral discomfort, such as spontaneous gingival bleeding and tooth pain, within seven days of travelling to a high-altitude location (Li and Liu., 2018). Therefore, we selected three time points for saliva collection: one day before travelling to a high altitude, seven days after arriving at a high altitude, and seven days after returning to a low altitude. This allowed us to analyze and compare the microbiomes at these three time points. Saliva sample collection is non-invasive, has a simple sampling process, and is low cost.

The diversity of the oral microbial community can reflect oral health. A previous study reported that the alpha diversity of the salivary microbial community in patients with dental caries was lower than that in healthy people (Belstrom et al., 2017). In our study, the alpha diversity of the salivary microbial community was lower in the altitude group than in the pre-altitude and post-altitude groups, which suggests that the prevalence of oral diseases may increase after high-altitude exposure. In addition, Liu et al. (Liu et al., 2021) reported that compared with a Tibetan population living in high-altitude areas, the alpha diversity of the salivary microbial community in a population living at ultrahigh altitudes decreased and that there was a significant negative correlation between altitude and the alpha diversity of the salivary community. AlShahrani et al. (AlShahrani et al., 2020) found lower diversity of oral microbes in orthodontic patients at high altitudes than in orthodontic patients at low altitudes; patients at high altitudes were more susceptible to periodontitis. Moreover, exposure to hypoxia has been shown to reduce the secretory function of salivary glands, resulting in the destruction of periodontal tissue (Terrizzi et al., 2018). We speculate that the alpha diversity of the salivary microbiome changes after high-altitude exposure because the colonization of oral bacteria is affected by the oxygen concentration.

In this study, anaerobic bacteria were dominant in the saliva of the altitude group and inhibited the colonization of aerobic bacteria. this may have led to the observed decrease in the alpha diversity of the salivary microbiome. Our results also showed that the beta diversity of the salivary microbial community changed after high-altitude exposure. Previous studies have found a positive correlation between the beta diversity of the salivary microbial community and altitude, and the beta diversity may thus be affected by altitude (Bhushan et al., 2019). Overall, these results indicate that acute altitude exposure affected the alpha and beta diversity of the salivary microbial community.

Changes in salivary symbiotic bacteria and increases in pathogenic bacteria may lead to the development of oral diseases (AlShahrani et al., 2020). Some studies have found that the relative abundance of Firmicutes decreased and that of Bacteroides increased with increased altitude in long-term Tibetan residents at different altitudes (Liu, et al. 2021). Bhushan et al. (Bhushan et al., 2019) found that the relative abundance of Firmicutes decreased and that of Bacteroides increased 25 days after subjects entered the Antarctic. However, in this study, Firmicutes increased significantly and Bacteroides decreased significantly after entering the plateau. Our study also found that the relative abundances of *Streptococcus* and *Veillonella* increased after arrival at high altitude. These results are similar to those of another study that found that the relative abundances of *Streptococcus* appeared to increase after arrival at high altitude (Zhao et al., 2022). These two genera are thought to be involved in the formation of oral plaque, which is implicated in the occurrence and development of dental caries and periodontal disease (Marsh and Zaura, 2017). However, Liu et al. (Liu et al., 2021) reported a decreased relative abundance of *Streptococcus* with altitude, and AlShahrani et al. (AlShahrani et al., 2020) found higher levels of *Streptococcus* and lower levels *of Veillonella* in orthodontic patients at high altitudes than in those at low altitudes. In this study, the relative abundances of *Prevotella*, *Porphyromonas*, and *Alloprevotella* decreased after arrival at high altitude. A previous study also showed that the relative abundance of *Prevotella* was higher at high altitudes (Xiao et al., 2012). There are no reports on the effect of altitude on *Alloprevotella* and *Porphyromonas* in the oral cavity. However, one study reported that the relative abundance of *Alloprevotella* in the stomach of cattle increased with increasing altitude (Fan et al., 2020). The above studies all suggest that high-altitude environments affect the abundance of common dominant bacteria, but some of the results are inconsistent with those of this study. These differences may be related to differences among studies in the time spent at high altitude.

Although this study did not find significant differences in the microbiota between subjects with swelling and aching of gum (or oral ulcer) and asymptomatic subjects. However, it clearly demonstrated the bacterial community and its symbiosis pattern in the saliva of the subjects under acute high-altitude exposure. As this was an observational study, we cannot be certain whether the change in bacteria was directly affected by the differences in the environment or the interactions among microorganisms. Therefore, we believe that acute altitude exposure affects oral microbial homeostasis to a certain extent. This finding is helpful to understand the potential

impact of acute high-altitude exposure on human oral health, but the specific mechanism needs to be further studied.

Oral symbiotic microbes are important for maintaining oral health. The mechanism by which symbiotes promote oral health is through resistance to colonization by pathogens, in which the symbiotes outperform disease-causing species in the colonization matrix and thus have little chance of integration by exogenous pathogens (Chalmers et al., 2008; Thurnheer and Belibasakis, 2018). It has been reported that *Streptococcus* and *Actinomyces* isolated from the oral environment of healthy people can inhibit the growth of *Porphyromonas gingivalis* (Sedghi et al., 2021). In our study, the correlation between *Streptococcus* and *Haemophilus*, *Veillonella*, and *Prevotella* changed after rapid high-altitude exposure. Therefore, this study found changes in the symbiotic relationship suggesting that oral health barriers are being broken.

Microorganisms not only participate in the body's immune response but also affect metabolic activity (Martin et al., 2010). Our data showed that the gene functional profile of the oral microorganisms was significantly affected by acute altitude exposure. Genes involved in carbohydrate metabolism were predicted to be upregulated, which may indicate that the oral microflora had increased energetic demands in the high-altitude environment. In addition, genes involved in the metabolism of cofactors and vitamins, including vitamin B6, were predicted to be downregulated in the altitude group. Studies have shown that a high level of vitamin B6 can help the human body eliminate reactive oxygen species, prevent oxidative stress damage, and adapt to a harsh external environment (Hellmann and Mooney, 2010). Moreover, other studies have shown that the vitamin B6 pathway is upregulated in the skin microbiota of high-altitude populations (Li et al., 2019) and in the oral microflora of an ultrahigh-altitude population compared with that of a high-altitude population (Liu et al., 2021). However, Monnoyer et al. found that in the extreme environment of saturation diving, the abundance of aerobic metabolic pathways in oral bacteria of divers increased relatively, while the anaerobic metabolic pathways decreased, mainly including energy metabolism, oxidative stress, and adenosine cobalamin synthesis (Monnoyer et al., 2021). This is mainly related to the survival and growth of bacteria in an oxygen-containing environment. Our results also found that the expression of genes related to vitamin metabolism was down-regulated after participants were rushed to high altitudes. So, we speculate that acute altitude exposure may affect the relative abundance of gene functions related to oral bacterial metabolic activity and may aggravate oxidative stress damage. These changes may affect the oral health of the subjects. As our results are based only on the predicted functions of the salivary microbiota, they do not represent the actual functions of the oral bacteria. In the future, further analysis of the roles of these genes in the oral cavities of people exposed to high altitudes is needed.

This study investigated the effects of acute high-altitude exposure, an environmental stressor, on the salivary microbiome. Since our study was only preliminary, we plan to use larger sample sizes, clinical indicators, and more accurate monitoring of subjects' physical activity in the future. Furthermore, future work should explore the relationship between the oral microbiota and oral health.

Conclusions

In conclusion, the diversity of the salivary microbiota decreased after acute high-altitude exposure, with an increase in the relative abundance of *Streptococcus* and *Veillonella* and a decrease in the relative abundance of *Prevotella*, *Porphyromonas*, and *Alloprevotella*. In addition, the correlation between bacteria genera changed. Bacterial metabolic functions include an increase in the relative abundance of carbohydrate metabolism gene functions and a decrease in the relative abundance of coenzyme and vitamin metabolism gene functions. These results advance our understanding of the salivary microbiota at high altitudes and its influence on oral diseases. In the future, we will further explore the specific mechanism of the effect of acute altitude exposure on the homeostasis of oral microorganisms.

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Figure 1

Comparisons of microbial community structure among three groups.

Venn diagram of OTU shared between the different groups (A). Species accumulation box diagram of saliva samples(B). Weighted UniFrac-PCoA of salivary microbiota from the three groups. PC1 explained 40.90% of the variation observed, and PC2 explained 23.98% of the variation(C). Analysis of similarity (ANOSIM) of community structure differences among groups.Ordinate shows the rank of the distance between samples.Between in the abscissa is the result Between two groups, and the other two are the results within their respective groups.The comparison between any two groups found that R > 0,P< 0.05, indicating that the difference between groups is greater than that within groups, the difference is statistically significant(Figure1D:P=0.03, R=0.1379. Figure1E:P=0.002, R=0.2127. Figure1F: P=0.004, R=0.2452).

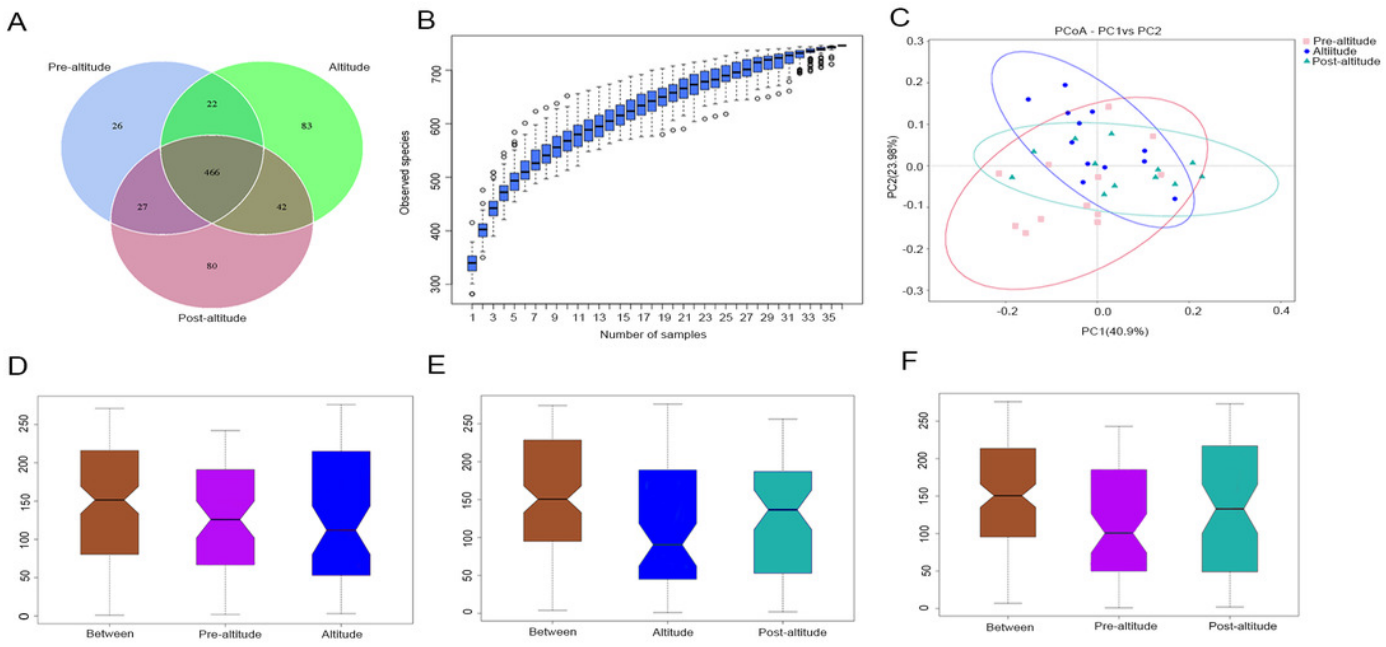

Figure 2

Bacterial Abundance and Distribution.

Distribution of the ten predominant bacteria at phylum level(A). The predominant phyla showing more than 1% of the mean relative abundance in the pre-altitude, altitude, and post-altitude groups are shown (B, C, D). Distribution of the ten predominant bacteria at genus level(E). The predominant genus showing more than 1% of the mean relative abundance in the pre-altitude, altitude, and post-altitude groups are shown (F, G, H). Statistically significant differences are marked with *P*-values.

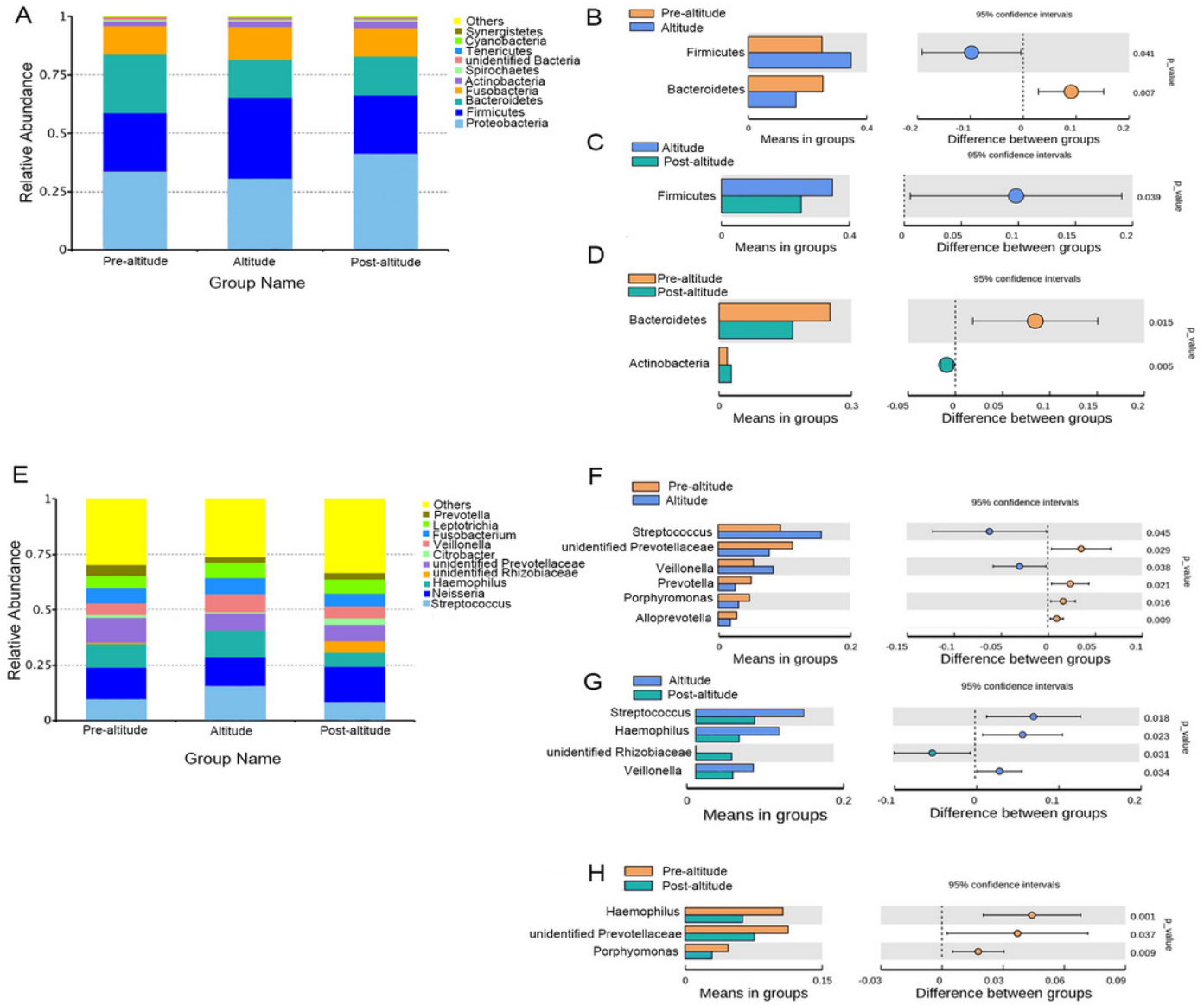
A
Relative Abundance
Group Name
Pre-altitude
Altitude
Post-altitude
Others
Synergistetes
Cyanobacteria
Tenericutes
unidentified Bacteria
Spirochaetes
Actinobacteria
Fusobacteria
Bacteroidetes
Firmicutes
Proteobacteria
B
Pre-altitude
Altitude
Firmicutes
Bacteroidetes
Means in groups
95% confidence intervals
Difference between groups
p_value
C
Altitude
Post-altitude
Firmicutes
D
Pre-altitude
Post-altitude
Bacteroidetes
Actinobacteria
E
Relative Abundance
Group Name
Others
Prevotella
Leptotrichia
Fusobacterium
Veillonella
Citrobacter
unidentified Prevotellaceae
unidentified Rhizobiaceae
Haemophilus
Neisseria
Streptococcus
F
Streptococcus
unidentified Prevotellaceae
Veillonella
Prevotella
Porphyromonas
Alloprevotella
G
Haemophilus
unidentified Rhizobiaceae
H
Porphyomonas

Figure 3

PICRUSt Function Predictions.

Distributions of the top ten predicted gene functions of the salivary microbiota in the three groups are shown. KEGG Pathways Level 1(A), KEGG Pathways Level 2(B). A comparison of predicted gene functions of salivary microbiota between the pre-altitude and altitude groups at KEGG Pathways level 3. Statistically significant P-values are shown in Figure(C).

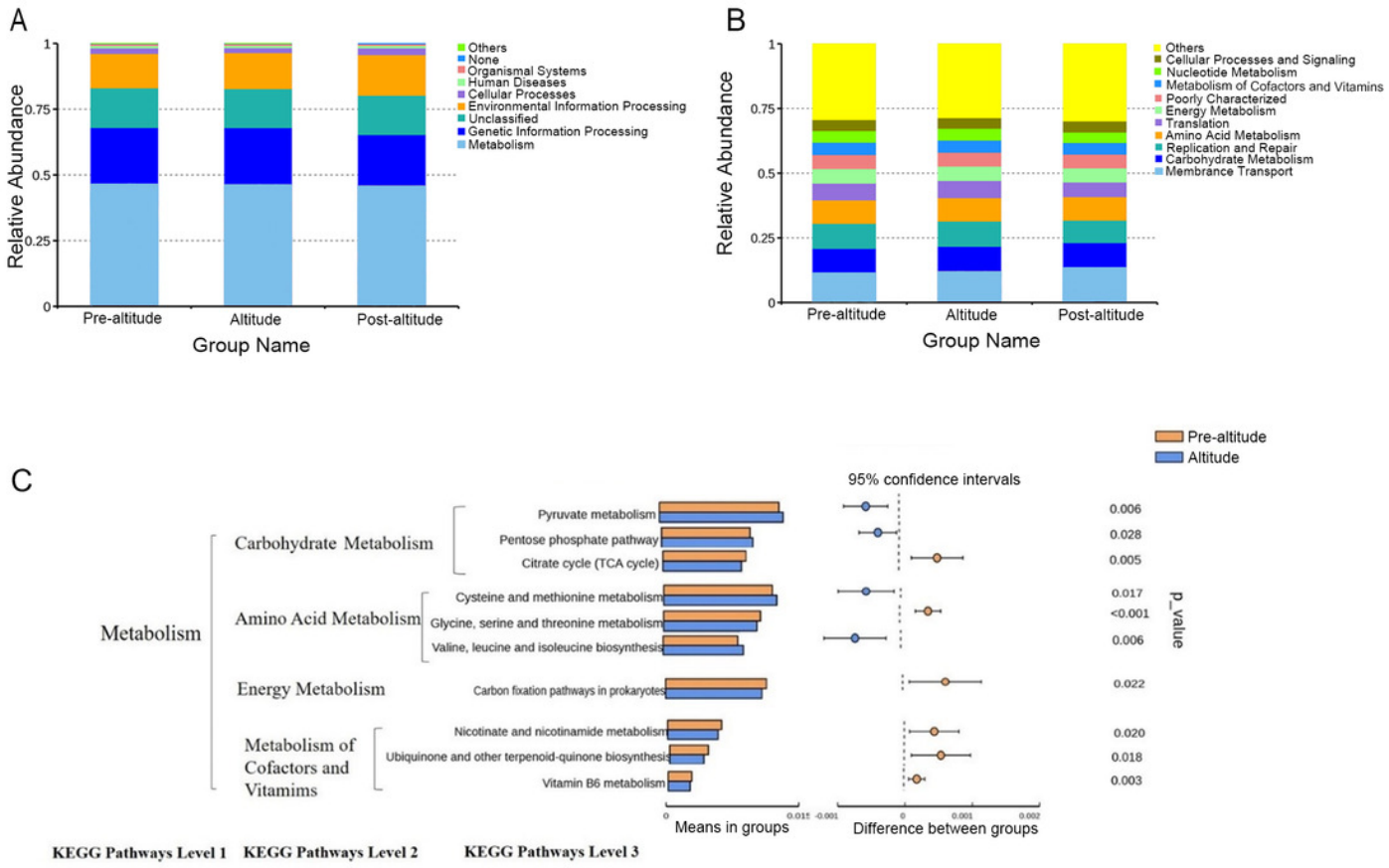

Table 1(on next page)

The assembly results from the saliva samples.

(1) Raw PE indicates the first data read out by the sequencing platform; (2) Raw tags refers to the sequence after splicing primer fragments from the original data; (3) Clean tags is a sequence with high quality and appropriate length obtained by filtering raw tags; (4) Effective tags refers to the sequences in clean tags that do not have chimeras and can be used for in-depth analysis; (5) Effective % refers to the ratio of effective data to the original number of offline users %. (6) Min length refers to the minimum length of valid data; (7) Max length refers to the maximum length of valid data; (8) Average length refers to the average length of valid data.

Table 1 The assembly results from the saliva samples.

Sample Number	Raw PE	Raw Tags	Clean Tags	Effective Tags	Effective (%)	Min length (bp)	Max length (bp)	Average Length (bp)
36	3138051	2944826	2802821	2298989	73.64%	418	425	422

(1) Raw PE indicates the first data read out by the sequencing platform; (2) Raw tags refers to the sequence after splicing primer fragments from the original data; (3) Clean tags is a sequence with high quality and appropriate length obtained by filtering raw tags; (4) Effective tags refers to the sequences in clean tags that do not have chimeras and can be used for in-depth analysis; (5) Effective % refers to the ratio of effective data to the original number of offline users %. (6) Min length refers to the minimum length of valid data; (7) Max length refers to the maximum length of valid data; (8) Average length refers to the average length of valid data.

Table 2(on next page)

Bacterial alpha diversity indices for saliva samples in each group.

[a]Shannon index between Altitude and Post-altitude group was statistically significant different (*P*=0.024). [b]Shannon index between Pre-altitude and Altitude group was statistically significant different (*P*=0.025). [c]Inverse Simpson index between Altitude and Post-altitude group was statistically significant different (*P*=0.022). [d]Inverse Simpson index between Pre-altitude and Altitude group was statistically significant different (*P*=0.007). [e]Simpson even index between Pre-altitude and Altitude group was statistically significant different (*P*= 0.004).

Table 2. Bacterial alpha diversity indices for saliva samples in each group

Group	Chao1		ACE		Shannon		Inverse Simpson		Simpson even		Good's coverage	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Pre-altitude	361.64	4.07	362.62	4.70	5.75[b]	0.08	26.40[d]	1.74	0.08[e]	0	0.99	0
Altitude	359.44	11.03	363.40	11.59	5.42[ab]	0.11	19.31[cd]	1.56	0.06[e]	0	0.99	0
Post-altitude	375.43	6.95	379.19	7.35	5.74[a]	0.07	25.28[c]	1.72	0.07	0	0.99	0

[a]Shannon index between Altitude and Post-altitude group was statistically significant different (P = 0.024). [b]Shannon index between Pre-altitude and Altitude group was statistically significant different (P=0.025). [c]Inverse Simpson index between Altitude and Post-altitude group was statistically significant different (P=0.022). [d]Inverse Simpson index between Pre-altitude and Altitude group was statistically significant different (P=0.007). [e]Simpson even index between Pre-altitude and Altitude group was statistically significant different (P= 0.004).