Succession of the Gut Microbiome in the Tibetan Population of the Minjiang River Basin

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Research

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Abstract

Background: Tibetans are one of the oldest ethnic groups in China and South Asia. Tibetans have a unique lifestyle and a long history, which leads to the particularity of the composition and function of their gut microflora. Tibetans in the Minjiang River basin have gradually increased their migration to the Chengdu Plain in recent years. Based on the analysis of 1059 Tibetans in the Minjiang River basin at an altitude of 500-4001 m, we analyzed the characteristics of the gut microbiome and further elaborated the main factors affecting the succession of the gut microbiome in the Tibetan population.

Results: Our study found that the dominant phyla of the Tibetan population were Bacteroidetes and Firmicutes, and the main genera were *Prevotella* and *Bacteroides*. To further study the factors affecting the gut microbial composition of the Tibetan population, 115 total parameters of 7 categories were evaluated. The results showed that altitude was the most important factor affecting the variation in the microbial community in the Tibetan population, and the change in altitude promoted the succession of the gut microbial community. In the process of migration from high altitudes to the plain, the gut microbial composition of late immigrants was similar to that of plateau aborigines, while that of early immigrants was similar to that of plain aborigines. Migration to Tibet is related to the loss of indigenous gut microbial community species. In addition, from low altitude to high altitude, the similarity of the microbial community with the high-altitude population increased with the reproduction of offspring after marriage. Changes in these microbials will affect the metabolism, disease incidence and cell function of the Tibetan population. The other two sets (AGP and Z208) of altitude data also showed the impact of altitude on the microbial community.

Conclusions: This is the first large-scale study on the factors influencing the gut microflora in a Tibetan population. Our study confirmed that altitude change is the most important factor affecting the distribution of the microflora in the Tibetan population and provided abundant and unique data to explore the interaction of impact parameter-gut microbiome-host function and disease.

Background

The gut microflora is the largest and most complex microecosystem in the human body [1, 2]. These microorganisms play important roles in digestion, vitamin synthesis, and immune system functioning. Together with the intestinal mucosa, the gut microflora constitutes an important physiological barrier against the invasion of pathogens [3, 4]. The function of the intestinal microbiota depends largely on its composition [3, 4]. Humans and bacteria have a balanced symbiotic relationship. Microbial taxa and abundances are in a dynamic balance and are influenced by environmental conditions, host diet, and genetic factors [5, 6].

A number of studies have shown that changes in the structure of the gut microflora can lead to metabolic syndrome [7, 8]. Recent clinical studies have found that the transplantation of the gut microflora can treat a variety of conditions, including Crohn's disease, ulcerative colitis, type 2 diabetes, antibiotic resistance,

alopecia, functional gastrointestinal diseases and neuropsychiatric diseases, such as Parkinson's syndrome, along with ameliorating the effects of antibiotic resistance [9–13]. Fecal microflora transplantation can also affect the efficacy of tumor immunotherapy [14–16].

Despite high similarity in the human gut microbiota among populations, different ethnic groups exhibit differences in the precise composition of the gut microflora. In a comparative study involving 173 Caucasian infants and 182 South Asian infants, ethnicity was an independent predictor of the intestinal microbial composition of infants; for example, *Bacillus* and *Lactobacillus* were abundant in South Asian infants, while *Fusobacterium* was abundant in Caucasian infants [17]. In addition, some studies have confirmed that there are differences in the gut microflora between Tibetan and Han populations [18, 19].

The Human Microbiome Project has produced increased information about intestinal microbes. However, little is known about the intestinal microbes of the Tibetan population in China. The Tibetan population, one of 56 ethnic minorities in China, mainly resides in the Tibetan autonomous region, the Qinghai Province, and the western Sichuan Province and experiences unique environmental and cultural conditions. The Minjiang River basin, with the largest concentration of Tibetan people in Sichuan, has an average altitude of more than 3,000 m. An increase in altitude corresponds to decreases in atmospheric pressure and oxygen partial pressure. The harsh environment of the plateau as well as the unique language and culture explain the relatively simple genetic background in the population. The high altitude of the plateau confers specific environmental characteristics, including low oxygen, low atmospheric pressure, and high radiation, as well as unique cultural, lifestyle, and dietary habits in Tibetan areas, all of which have been shown to affect the microbiome [20–22]. With a history of over 25,000 years in the area, indigenous Tibetans have adapted to the plateau and therefore serve as a good model for exploring the effects of the environment on the gut microbiota [23].

Migration from non-Western countries to the United States is associated with a reduction in gut microbiome diversity and function and an increased predisposition to metabolic diseases [24]. However, the factors that influence changes in the gut microbiome in the Tibetan population are unclear. The Tibetan population in areas surrounding the Minjiang River and tributaries recently migrated to the Chengdu Plain. This change in environmental conditions, cultural practices, and other factors has substantially altered the lifestyle of this Tibetan population. Tibetans at high altitudes mainly eat beef, milk, and highland barley, while populations at low altitudes mainly consume pork and rice as staple foods. In addition, the influence of changes in the Tibetan gut microbiome on disease incidence and physiological function needs to be further explored.

In this study, we assessed the characteristics of the intestinal microflora of the Tibetan population. In particular, we collected fecal samples from 1059 native Tibetan individuals living at altitudes of 500–4001 m. We evaluated environmental conditions, dietary habits, disease statuses, and other factors and performed blood biochemical tests. We further assessed the effects of migration on the intestinal flora using 170 Tibetan individuals who migrated from altitudes of over 1000 m to the plain, together with 36 additional migrants who migrated from the plain to the plateau.

Methods

Subject selection and sampling

Volunteers (natural crowds) were recruited from populations in the Ngawa Tibetan Autonomous Region and the Chengdu Plain of Sichuan Province. The individuals resided in cities or towns along the main Minjiang River and tributaries, including Hongyuan, Barkam, Jinchuan, Heishui, Songpan, Wenchuan, Dujiangyan, and Chengdu (Fig. 1a), at altitudes of 500–4001 m. A total of 1251 participants were enrolled (sample information is listed in Table S1), and fecal samples from 1059 native Tibetan individuals were further analyzed. To study the effect of migration, 170 participants were recruited who migrated from the Ngawa area to the Chengdu Plain, together with 36 additional migrants, including those 1) born on the plain who moved to the plateau (Immigrant 1; n = 9), 2) born on the plateau with parents born on the plain (Immigrant 2; n = 20), and 3) born on the plateau with grandparents or ancestors born on the plain (Immigrant 3; n = 7). A questionnaire survey was completed by all volunteers regarding basic demographic information (age, sex, birthplace, place of residence, ethnicity, etc.), health status (digestive tract diseases, type 2 diabetes, mental health, genetic diseases, etc.), diet (staple food, dietary intake, drinking status, consumption of coffee, tea, yogurt, etc.), and exercise (daily physical activity, exercise frequency, etc.). The height, weight, and blood pressure of all participants were recorded. Fecal samples were freshly collected and transferred within 6 hours to a -80°C freezer until use. Peripheral fasting venous blood was collected for routine blood tests (hemoglobin, erythrocyte, white blood cell, and blood platelet counts) and biochemical tests (liver and kidney function, blood glucose, and lipid levels). Standardized procedures were applied at all collection sites by the same trained personnel. Staff and procedures were regularly checked for quality throughout the data collection period. This experiment was approved by the Ethics Committee of Chengdu Medical College (No. 2017009). Informed consent was obtained from all participants.

Metadata collection

Based on questionnaire surveys and blood tests, metadata were obtained, including sociodemographic characteristics, anthropometric characteristics, and information about lifestyle, diet, drug use, diseases, and biochemical parameters. In total, 115 factors were screened and further divided into the following 7 categories: basic, environment, drug use, disease, diet, sport, and biochemical parameters (Table S2).

Sequencing and taxonomic profiling

DNA samples were quantified using a Qubit 2.0 Fluorometer. The V3–V4 hypervariable regions of the 16S rRNA gene from bacteria and archaea were amplified using the 341F/806R primer set. Sequencing was performed with a 2 × 250 paired-end (PE) configuration using the Illumina HiSeq platform. The raw PE reads were merged using FLASH (Version 1.2.7) [25], and low-quality and polyclonal sequences were filtered using QIIME (Version 1.9.1) [26]. By further comparison with the Gold database, chimeric reads were removed using Usearch (Version 8.1.1861) [27]. The resulting reads for each sample were clustered into operational taxonomic units (OTUs) at the level of 97% similarity using QIIME (Version 1.9.1). A

representative sequence for each OTU was selected, and annotation was performed using QIIME (Version 1.9.1) based on the GreenGenes database (Version 13.8) [28]. After random rarefaction of sequences to the minimal number of reads in all samples, microbial composition at each taxonomic level was evaluated using QIIME (Version 1.9.1). The dataset supporting the results of this article has been deposited in the EMBL European Nucleotide Archive (ENA) under BioProject accession code: PRJEB13870.

Multivariate association

Missing values from the metadata were imputed using the mice package in R (Version 3.3.3) [29], and collinear variables were detected by a Pearson correlation analysis (Pearson $|r| > 0.8$). Correlations between clinical parameters (categorical or numerical) and microbiota community ordination generated by nonmetric multidimensional scaling (NMDS) based on Bray–Curtis distances were calculated, as previously described [30]. For collinear pairs, variables that were weakly correlated with the microbial community were filtered. Envfit was used in the vegan R package to conduct the MANOVA and to estimate linear correlations of categorical and numerical variables of the microbiota. Fifty factors were selected as significant determinants (10,000 permutations; $P < 0.05$; adjusted $P < 0.05$) of the microbial community, and the effect size (r-value) for each factor was determined. The combined effect sizes for the 7 categories (basic, environment, disease, diet, biochemical, sport, and drug use) were also generated. After Bray–Curtis distance matrixes of sub-metadata and microbiota community data were generated, the correlation between the two distance matrixes was calculated ($|r|$, combined effect size) by the Mantel test in the vegan R package. In addition, clinical variables with significant contributions to core- and unique genus-level community ordination were analyzed. Genera observed in more than 90% of samples were defined as the core microbiota, and genera detected in less than 10% of subjects were defined as unique. The taxonomic tree was visualized using GraPhlAn (Version 1.1.3) [31]. The Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg) database was also used. Based on the KEGG pathway analyses, the differentially gut microbiomes were annotated and their functions determined.

Datasets

Gut microbiota data available in public databases and the literature, including the AGP (American Gut Project Database), the LLD (LifeLines-DEEP Database), and the flora resources published by Lan [32] (referred to as S208) and Zhang [22] (referred to as S314), were compared with our data set (referred to as Zang). Data were divided according to ethnic groups or regions to analyze the differences in the composition of the intestinal flora and specific characteristics of populations. To validate the impact of altitude on the gut microbial community, two databases with altitude information (AGP and S208) were used to obtain information for 1244 subjects and 208 Tibetans from six locations in China: Gannan, Gangcha, Tianzhu, Hongyuan, Lhasa, and Nagqu.

Statistical analyses

Alpha diversity indices (i.e., the observed OTUs, Chao1 index, Shannon index, and Simpson index) were measured using QIIME (Version 1.9.1). To quantify differences (beta diversity) between samples, the phylogeny-based weighted and unweighted UniFrac distances between all pairs of samples were calculated using QIIME. Principal coordinate analysis (PCoA) and NMDS were used to visualize the differences between samples with the ade4 R package.

Enterotyping was performed as described previously [33]. Briefly, all samples were analyzed by the partitioning around medoids clustering method based on the Jensen–Shannon distances for genera abundances. The optimal number of clusters was estimated using the Calinski-Harabasz (CH) index (where higher values are better). Only genera detected in at least 10% of samples were included in the analysis.

To determine significant associations between clinical variables (categorical or numerical) and genera, a multivariate association analysis was performed using MaAsLin [34]. Spearman's correlation coefficients for relationships between continuous variables and microbiota were determined. The differences in alpha diversity indices, genera, and variables between groups were tested by the Wilcoxon rank sum test or the Kruskal–Wallis test, and *P*-values were calibrated by the Benjamin method. Significance was defined as an adjusted *P*-value of < 0.05.

Results

Altitude affects the variation in microbial groups in the Tibetan population

Individuals of the native Tibetan population of the main Minjiang River and tributaries in Sichuan Province at altitudes of 500–4001 m were recruited (Fig. 1a). PCoA data for Tibetan samples indicated that *Bacteroidetes* and *Firmicutes* were the two most abundant phyla (Figure S1a). Five core genera were present in Tibetan individuals, *Prevotella* (22.06%), *Bacteroides* (9.08%), *Faecalibacterium* (3.54%), *Lachnospira* (1.43%), and *Ruminococcus* (1.13%), accounting for 32.75% of the total sequences, and within *Bacteroidetes*, the core species mainly belonged to the order *Bacteroidales* and class *Bacteroidia* (Figure S1b). The community richness and community diversity of the microbiome in the Tibetan population in the region mostly consistent with previous literature reports (Figure S1c). Based on the CH index, the Tibetan samples were assigned to enterotype 1 (*Prevotella*) and enterotype 2 (*Bacteroides*) (Figure S1e). To assess whether the flora of the Tibetan population is unique, we compared our dataset Zang with the datasets of LLD (Dutch, 1010 samples), AGP (American, 1313 samples), S314 (healthy young Chinese, 314 samples) and S208 (Tibetan, 208 samples). The 3D map of the flora distribution (Figure S2) indicated that our dataset Zang and dataset S208 of the Tibetan population showed high similarity and were distinguished from the other three datasets, reflecting the specificity of the microflora of the Tibetan population.

To further study the factors affecting the composition of the gut microbes in the Tibetan population, 115 total parameters in seven broad categories were evaluated. Out of these categories, drug use and basic population parameters were the main factors affecting the overall flora composition (Fig. 1b). With respect to the overall flora and the core flora, altitude exerted the strongest effect, followed by age, antibiotic drug use (within 3 months), fried food, and platelet distribution width (PDW) (Fig. 1c and Figure S3a). The unique microbiota was greatly affected by the clinical indicator alanine aminotransferase (ALT) (Figure S3c). In terms of the seven categories, drug use was the most important determinant of the core flora, consistent with previous findings (Figure S3b) [35]. For the unique microbiota, the environment category had the greatest impact (Figure S3d). Furthermore, 38 genera significantly correlated with altitude were screened using MaAsLin ($P < 0.01$). *Firmicutes* and *Proteobacteria* were the dominant phyla related to altitude (Fig. 1d).

These results indicated that altitude was indeed the most important factor affecting the gut microbiome in Tibetan populations and further supported the uniqueness of the microflora in individuals living in Tibetan areas.

Altitude migration drives gut microbiome succession in the Tibetan population

The relationship between altitude and species composition in the Tibetan population was explored to further study successional patterns. The overall composition of the Tibetan microflora varied across altitudes based on NMDS2 analysis (Figure S4a). The abundances of *Megamonas, Bacteroides, Prevotella, Fusobacterium*, and *Lachnospira* decreased as the altitude increased and were defined as negative with altitude (Figure S4a). In contrast, the abundances of *Coprococcus, Dialister, Succinivibrio, Megasphaera*, and [*Prevotella*] increased as the altitude increased, defined as positive with altitude (Figure S4a). Eight genera were further selected to analyze the differences in altitude adaptability (Figure S4b). *Klebsiella* decreased as the altitude increased, while *Lachnospira* and *Megamonas* showed good adaptability to high altitudes and maintained relatively high abundances (Figure S4b). It should be pointed out that the abundance of *Lachnospira* increased significantly ($p < 0.05$) at altitudes of 1,000–2,000 m and then decreased as altitude increased. *Megamonas* showed a higher abundance at 1,000–3,000 m, with a significant ($p < 0.05$) decrease in abundance at altitudes above 3,000 m. A steady increase in the abundance of *Oscillospira* was detected with increasing altitude, indicating good adaptability to high altitudes. *Clostridium, Lachnobacterium*, and *Akkermansia* all showed relatively stable abundances, except at altitudes exceeding 3,000 m (Figure S4b). Taken together, these results show that alpha diversity (observed OTUs, Chao1 index, Shannon index, and Simpson index) was positively associated with altitude above 1,000 m (Figure S4c). Spearman's correlation analyses were used to evaluate relationships between genera and altitude (Figure S5a). *Clostridium*, *Oscillospira*, *WAL_1855D*, *Succinivibrio*, and *CF231* were positively correlated with altitude, while *Bacteroides*, *Trabulsiella*, *Serratia*, *Erwinia*, and *Citrobacter* were negatively correlated with altitude. To eliminate bias due to the uneven distribution of samples at different altitudes, the relative abundances of these genera were acquired from random sampling and transformed into Z-scores (Figure S5b).

Next, we hypothesized that the intestinal flora was influenced by migration. A total of 776 Tibetan individuals were divided into three groups (Fig. 2a): plateau-born (born and living on the plateau, n = 586), plain-born (born and living on the plain, n = 20) and plateau-Trans (born on the plateau and moved to the plain, n = 170). An NMDS2 plot was generated based on the genera profile and the time of migration to the plain and was then used for a linear fitting analysis. As shown in Fig. 2b, the gut microbiome composition differed with respect to migration time. In particular, late migrants had gut microbial communities that were similar to those of the indigenous population on the plateau, while early migrants had microbial communities that were similar to those of the native population on the plain. In terms of alpha diversity (Fig. 2c), earlier migrants exhibited higher levels of microbial diversity, consistent with that of samples from the plain. Standard deviations (SD) in prevalence in different groups revealed convergent losses in diversity from the plateau to the plain, indicating that the time of migration was correlated with the loss of alpha diversity. The ratio of *Bacteroides* to *Prevotella* is an important indicator of the status of bacteria [36]; accordingly, we analyzed the distribution of the log-normalized *Bacteroides*-to-*Prevotella* (B/P) ratio in different groups (Fig. 2d). The longer the time since migration, the higher the B/P ratio and the closer the ratio was to that of the indigenous population on the plains. Furthermore, Spearman's correlation analysis of the overall distribution of genera showed that the genus type was associated with the year of migration. Based on abundance profiles transformed into Z-scores (Fig. 2e), species with significantly higher abundances in the earlier migrants were also more abundant in the population on the plain.

In brief, altitude drives gut microbiome succession in the Tibetan population, and Tibetan migration is associated with the loss of diversity in the gut microbiome.

The immigration from the plain to the plateau is related to microbiome succession

In addition, to study the effect of migration, 170 participants were recruited who migrated from the Ngawa area to the Chengdu Plain, together with 36 additional migrants, including those born on the plain who moved to the plateau (Immigrant 1; n = 9), those born on the plateau with parents born on the plain (Immigrant 2; n = 20), and those born on the plateau with grandparents or ancestors born on the plain (Immigrant 3; n = 7) (Fig. 3a). Based on the genera profile used to determine the overall microflora structure, the flora in each generation of migrants was different from that of samples from the plain and differed with respect to ethnicity (Fig. 3b). We obtained six genera in which their abundance was significantly correlated with migration by the MaAsLin method. *Lachnospira* had a high abundance in samples from the plain, and the abundance gradually decreased across generations. *Klebsiella* had a high abundance only in the plains group (Fig. 3c). In terms of alpha diversity in plateau-born, plain-born, and 36 plain-to-plateau individuals (Fig. 3d), the SD in the different groups revealed convergence in variation from the plain to the plateau. In general, the shorter the time since migration, the smaller the difference in sample diversity, and the longer the migration time, the greater the difference in sample diversity (Fig. 3d).

Altitude migration could affect the diversity of the Tibetan gut microbiome To further explore the influence of altitude migration on the gut microbiome of Tibetan population, we analyzed the change in core flora in different periods from high altitude to low altitude (year of migration), and the core flora changed in different generations from low altitude to high altitude (immigration). Through correlation analysis, the correlation network showed that 15 bacterial communities changed in the process of altitude migration. Among them, *Lachnospira, Bacteroides* and *Clostridium 2* were negatively correlated with altitude migration, while the changes in *Lactobacillus*, *[Prevotella]*, *Dialister*, *Prevotella*, *Succenivibrio*, *Catenibacterium*, *Collinsella*, *[Euberium]*, *CF231*, *Slackia*, *Oxalobacter*, and *Dehalobacterium* were positively correlated with altitude migration (Fig. 4a).

We further analyzed the influence of 115 parameters on the evolution of the gut microbiome in the process of altitude migration. For people migrating from the plateau to the plain, altitude and fried food significantly contributed to the diversification of the Chao1 index (Fig. 4b), and the drinking type significantly contributed to taxonomic composition of the gut microbiome (Fig. 4c). For people migrating from the plain to the plateau, altitude and fried food also had the greatest impact on the Chao1 index (Fig. 4b), and altitude significantly contributed to taxonomic composition (Fig. 4c).

Altitude migration could affect the physiological function and disease incidence in the Tibetan population

Changes in the gut microbiome are related to physiological function and disease incidence in the host. To study the influence of altitude migration on the physiological function and disease incidence in the Tibetan population, we conducted Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. Predicted KEGG pathways revealed that metabolism, signal transduction, transcription, excretory and nervous system pathways were positively correlated with the year of migration from the plateau to the plain (Fig. 5a). Cancer, immune and digestive systems, cell growth and death, replication and repair pathways were negatively correlated with the year of migration from the plateau to the plain (Fig. 5a). We further analyzed the influence of gut microbial changes on the Tibetan population from the plain to the plateau. KEGG analysis showed that metabolism, signal transduction, transcription, excretory and nervous system, poorly characterized and cellular processes and signaling pathways were negatively correlated with the generations that immigrated from the plain to the plateau (Fig. 5b and Figure S6), while cell growth and death, replication and repair, translation, folding sorting and degradation, cancer and digestive system pathways were positively correlated with the generations that immigrated from the plain to the plateau (Fig. 5b and Figure S6). The above analysis showed that with the migration between altitudes, the changes in microorganisms mainly affected the metabolism, signal transduction, transcription, cancer, digestive system, cell growth and death and replication and repair pathways of the Tibetan population.

Construction of the network of impact parameters, gut microbiome and function

Gram staining of bacteria is one of the important methods used to distinguish bacterial species, which could guide the diagnosis and treatment of diseases [37]. Therefore, we analyzed the changes in the abundance of gram-positive and gram-negative bacteria in the process of altitude migration. Among the people who migrated from the plateau to the plain, with a shorter migration time, there were more gram-negative bacteria and less gram-positive bacteria, the pathogenic potential of the bacteria was stronger, and the content of mobile elements was lower (Fig. 6a). Because the change in altitude mainly leads to a change in environmental oxygen content, we further analyzed the changes in the abundance of aerobic and anaerobic bacteria in the process of altitude migration. A shorter migration time led to less anaerobic bacteria, more facultative anaerobic bacteria and better the oxygen tolerance (Figure S7). In addition, for people who migrated from the plain to the plateau, a longer migration time led to a lower content of mobile components, an increased abundance of gram-negative bacteria and a decreased abundance of gram-positive bacteria (Figure S8).

Finally, we constructed a network of impact parameter-gut microbiome functions based on altitude and dietary factors, 11 bacterial communities that changed in abundance in the process of altitude migration and changes in the physiological functions associated with the gut microbiome. *Prevotella* was the most closely related to the relevant KEGG pathways, and the factors most related to the genus and metabolic pathways were altitude, along with the consumption of milk, tea, vegetables, fried food, and sweets (Fig. 6b).

Altitude could affect the composition of the gut microbiome in other ethnic groups

To analyze the effect of altitude on other populations, we compared our data with the two datasets AGP and S208, for which altitude information was available. We used PCoA plots based on Bray–Curtis dissimilarity at the genus level to depict the overall distribution of the intestinal flora at different altitudes. Under different data sets, altitude clearly distinguished the samples (Fig. 7a). Similarly, when we analyzed AGP and S208 separately, the high-altitude samples in AGP and S208 could be differentiated in the PCoA plots (Figure S9a, b). Based on Spearman's correlation coefficients, we identified correlations between the relative abundances of genera and altitude in the S208 (top) and Zang (bottom) datasets (Fig. 7b). In our dataset (Zang), *Bacteroides* was more abundant in low-altitude samples; however, in the S208 dataset, this taxon was more abundant at high altitudes. In addition, other taxa that were positively correlated with altitude in our dataset were also found in S208. All samples from the S208 dataset were clustered into enterotype 1 (*Prevotella*) and enterotype 2 (*Bacteroides*) by PCoA based on Jensen–Shannon distances (Figure S9c), consistent with the results for our dataset. In another Tibetan population (dataset S208), the core genera belonging to Bacteroidetes were *Prevotella* and *Bacteroides*, and in Firmicutes, the core genus was *Faecalibacterium* (Figure S9d). The core genera identified in S208 were also identified in our dataset, illustrating the similarity in the intestinal flora of Tibetan populations from different regions.

Discussion

This is the first large-scale study of the gut microflora of the Tibetan population in Ngawa. In the course of human history, civilization is often formed along a river. To evaluate microbial succession in the Tibetan population, we collected 1059 samples from the Ngawa Tibetan autonomous region and the Chendu Plain of Sichuan Province, from upstream to downstream along the Minjiang River and its tributaries. Our results showed that the gut microbiota of the Tibetan population was dominated by *Bacteroidetes* and *Firmicutes*. The flora distribution of the Tibetan population was distinguished from the other three datasets (LLD, AGP, S314). As the altitude increased, bacterial diversity increased, consistent with previous results [38]. Zhang et al. [22] studied the gut microbiome of 314 healthy young people from 7 ethnic groups in 20 villages and towns in 9 provinces of China and obtained nine core genera, *Phascolarctobacterium*, *Roseburia*, *Bacteroides*, *Blautia*, *Faecalibacterium*, *Clostridium*, *Subdoligranulum*, *Ruminococcus*, and *Coprococcus*. In comparison, the unique core genera in the Tibetan population in Ngawa were *Prevotella* and *Lachnospira*. *Prevotella* mainly participates in the metabolism of carbohydrates and plant proteins as well as short-chain fatty acid production [39]. Many *Lachnospira* strains produce butyrate, which plays a crucial role in the maintenance of human gut health [40]. These bacteria produce short-chain fatty acids, which can act as anti-inflammatory agents [40]. This may explain how Tibetans with a low dietary fiber intake maintain gut health. These bacteria may be related to adaptation to the low-oxygen environment and may be beneficial to Tibetans in high-altitude areas. The Tibetan microflora can be divided into two enterotypes, as observed in both our dataset (Zang) and dataset S208. A previous study reported that the human gut microbiota can be divided into three enterotypes, *Prevotella*, *Bacteroides* and *Ruminococcus* [41]. The gut microbiota of the Tibetan population mainly consisted of *Prevotella* and *Bacteroides*, while *Ruminococcus* accounted for only a small portion of the sequence reads (~ 1%). We speculate that this can be explained through the unique diet of Tibetans. *Ruminococcus* is mainly related to the digestion of carbohydrates, such as sugar, starch, and potato [42]. Tibetans prefer high-protein, high-fat, and high-fiber foods, such as highland barley, while their carbohydrate consumption is relatively low.

Wang W et al [43] studied individuals from six regions of the plateau and found that location, altitude, BMI, age, and other factors affected the gut microbial composition. However, the main determinant of the flora was not established, and the study involved a small sample size. Deschasaux [44] studied the gut flora of more than 2,000 individuals of different ethnicities in the same city and showed that ethnicity contributes significantly to individual differences in the gut microbiome, independent of metabolic health. Accordingly, studies of human flora should consider ethnicity as a contributing factor. A study on the gut microbiome of more than 7,000 individuals in 14 districts of Guangdong Province found that regional factors have a significant impact on the flora [45].

Most Tibetans live in harsh environments of high altitude, low pressure and hypoxia. Under such challenging conditions, the correlation between gut microbiome determinants and physiological responses and clinical problems is very important. Our study confirmed that altitude had the greatest effect on the microbial composition. This is also reflected in the analysis of immigrants from the Qinghai-Tibetan Plateau and Han immigrants from the plain to the plateau [46]. The important influence of altitude on flora was further indicated by two other datasets, AGP and S208. High altitude is related to

low pressure, hypoxia, low temperature and high radiation. These factors all affect biological processes. Hypoxia can produce reactive oxygen species through the Grb2/EGFR/PTPN11 pathway, increase the expression of HIF-1α and iNOS, thin the intestinal mucosa, result in irregular and fewer epithelial cells, and decrease the number of tight junction proteins [47–48]. These changes can lead to mucosal barrier dysfunction. In addition, hypoxia can affect the intestinal mucosal immune system [49]. Low temperature can also affect the gut microbiome, which has a protective effect on obesity [50]. With regard to solar radiation, studies have proposed the intestinal-skin axis and have shown that skin exposure to radiation can regulate the gut microbiome [51, 52]. The effect of altitude on the gut flora may be the result of the above factors and reflects the adaptability of the gut microbiome. Among the genera with a positive correlation between abundance and altitude, *Clostridium* was previously identified by Adak et al. [53]. *Lachnobacterium*, *Akkermansia* and *Eubacterium rectale* are mucus-related microorganisms that are beneficial to human health [54]. Interestingly, Vangay et al found that the short-term response of the whole microbial community to migration varied from individual to individual, but microbial community changes in the main native and dominant groups in the United States began within 6 to 9 months of residence in the United States [24]. Soldiers who moved from plains to high places for 15 days showed a decrease in total oxygen demand and an increase in total anaerobes and facultative anaerobes [55]. At the same time, our study confirmed that during migration from high altitude to plains, the intestinal microbial composition of late immigrants was similar to that of plateau aborigines, while that of early immigrants was similar to that of plain aborigines. Migration to Tibet is related to the loss of indigenous intestinal microbial community species. In addition, after marriage, the similarity of the microbial community in the high-altitude area increased with the reproduction of offspring. Finally, our analysis of different datasets (Z208, USA) further suggests the important influence of altitude on the gut microbiome.

We confirmed that altitude is an important factor affecting the succession of the gut microbiome. We aimed to determine the effects of these changes on the metabolic functions in the Tibetan population. Our study found that in the migration process of the Tibetan population, changes in the gut microbiome were most related to metabolism, cell growth and death, signal transmission, cancer, the immune system and the digestive system. Alessia Visconti et al. showed that the gut microbiome was closely related to host systemic metabolism. The metabolic pathway was significantly correlated with 95% of the fecal metabolites, while the microbial species were related to 82% of the fecal metabolites. The carcinogenesis of colorectal cancer was significantly correlated with the gut microbiome. There is some evidence to indicate the association between that pathogenesis of *Fusobacterium nucleatum* and colorectal cancer [56, 57]. At the same time, the intestinal microflora plays a very important role in the treatment of tumors. The influence of intestinal microbiota on the immune checkpoint blocking reaction was initially studied in a mouse model. In 2015, a landmark paper was published in Science, which showed that the composition of intestinal microbiota can affect the immune checkpoint for cytotoxic T lymphocyte antigen-4 (CTLA-4) inhibitor response and death receptor 1 (PD-1) [15, 16]. Recent studies have shown that the gut microbiota is associated with immune system diseases, for example, *P. gingivalis* impacts the development of autoimmune diseases [58]. Hevia et al found that the ratio of intestinal

Pachymete/ *Bacteroides* decreased in patients with systemic lupus erythematosus, indicating that mucosal immune dysfunction in patients with systemic lupus erythematosus affects the intestinal microbiota [59]. *Bacteroides* found in fragile substances in the human gut play an active regulatory role in the human immune system [60]. These studies highlight the complex interaction between the gut microbiome and host function.

Gram staining of bacteria is one of the important methods used to distinguish bacterial species and can guide the diagnosis and treatment of diseases [37]. Our research found that a shorter migration time led to an increased abundance of gram-negative bacteria and a decreased abundance of gram-positive bacteria among people migrating from the plateau to the plain. This finding has good clinical guiding significance for the infection of Tibetan people and the choice of antibiotic.

Conclusion

Tibetans are one of the oldest ethnic groups in China and South Asia. As an important ethnic group in China, Tibetans have formed a unique lifestyle and customs to adapt to the harsh environment. Studying the composition of the gut microbiota of the Tibetan population can provide insight into differences in microbial colonization among regions and ethnic groups as well as the contributions of the unique adaptive lifestyle, customs, and dietary habits of Tibetans to the intestinal microecology. In contrast to the Tibetan population on the Qinghai-Tibet Plateau, Tibetans in the Minjiang River basin have gradually increased their migration to the Chengdu Plain in recent years. To further study the factors affecting the gut microbial composition of the Tibetan population, 115 total parameters of 7 categories were evaluated. Our study was the first large-scale study on the influencing factors of gut microflora in a Tibetan population. We indicated that altitude was indeed the most important factor affecting the gut microbiome in Tibetan populations and further supported the uniqueness of the microflora in Tibetan areas. The change in altitude promoted the succession of the gut microbial community. The other two datasets (AGP and Z208) that included altitude data also showed the impact of altitude on the microbial community. Furthermore, our study provided abundant and unique data to explore the interaction of impact parameter-gut microbiome-host function and disease incidence.

Abbreviations

NMDS: non-metric multi-dimensional scaling; AGP: American Gut Project Database; LLD: LifeLines-DEEP Database; PCoA: principal coordinate analysis; CH index: Calinski-Harabasz index; PDW: platelet distribution width; ALT: alanine aminotransferase; KEGG: Kyoto Encyclopedia of Genes and Genomes. CTLA-4: cytotoxic T lymphocyte antigen-4; PD-1: death receptor 1.

Declarations

Availability of data and materials

The datasets generated and/or analyzed during the current study has been deposited in the EMBL European Nucleotide Archive (ENA) under BioProject accession code PRJEB13870.

Ethics approval and consent to participate

This experiment was approved by the Ethics Committee of Chengdu Medical College (No.2017009). Informed consent was obtained from all participants.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Jun Li mainly contributes to design this study and written the original manuscript. Lin Sun and Chunfen Mo designed the study. Baijun Chen, Jing Liu, Xuemei Li, Lingmeng Song and Wen Yang responsible for the collection and processing of samples. Luo Zuo, Yan Zhou, Jingping Sun, Ling Qin and Feng He supervised and administered the project and provided funding. Li Deng mainly involved in the revision of the manuscript. Yuanqin Tang, Lin Yang, Lesiji Kang, Yonghua He and Songbo Wang involved in sample storage and processing. Junru Chen and Xianyue Wang compiled and curated the data and performed bioinformatic analysis. Xiaofeng Qin and Xiaoan Li were responsible for project implementation and quality control. All co-authors contributed substantially to manuscript revisions. All authors read and approved the final manuscript.

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Figures

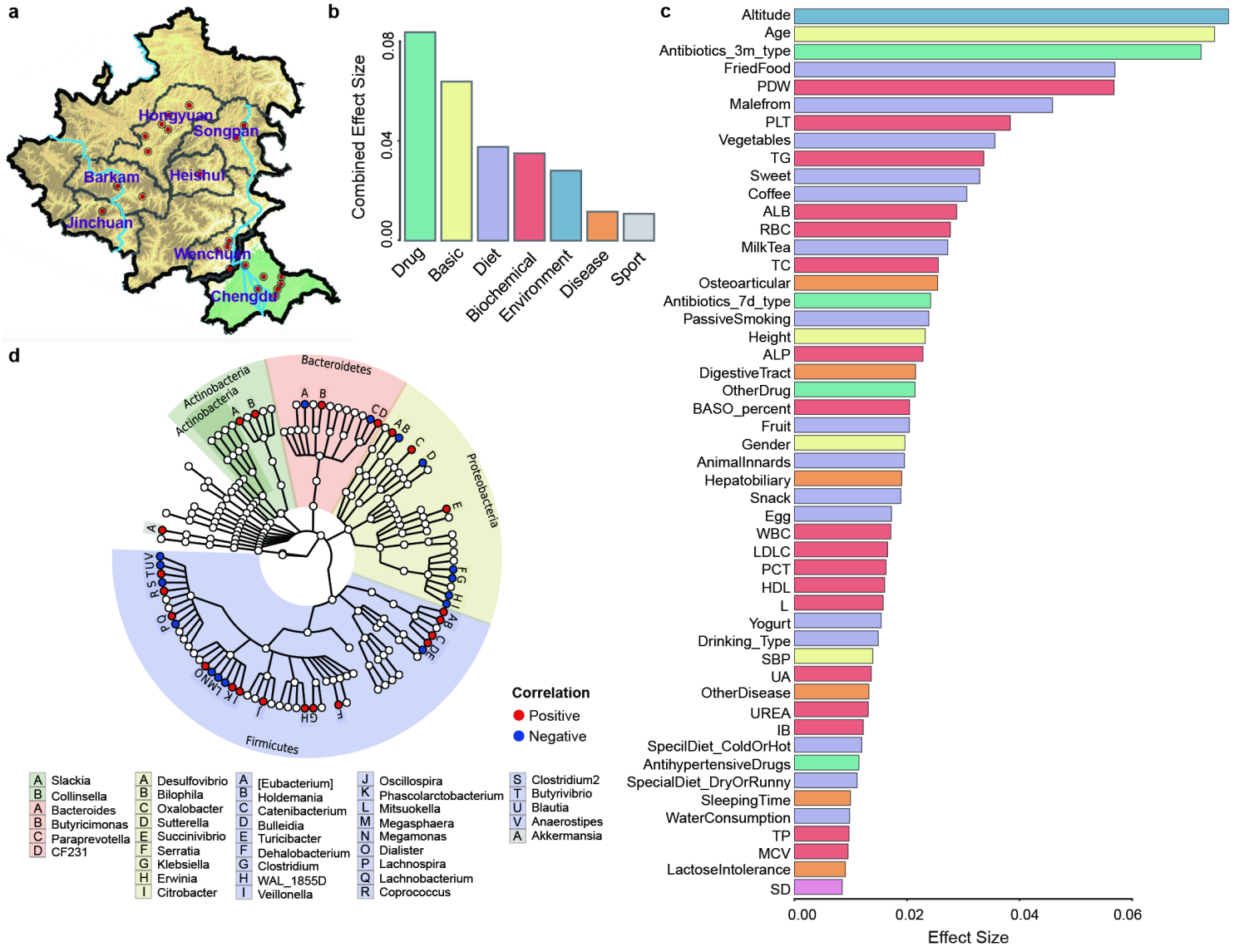

Figure 1

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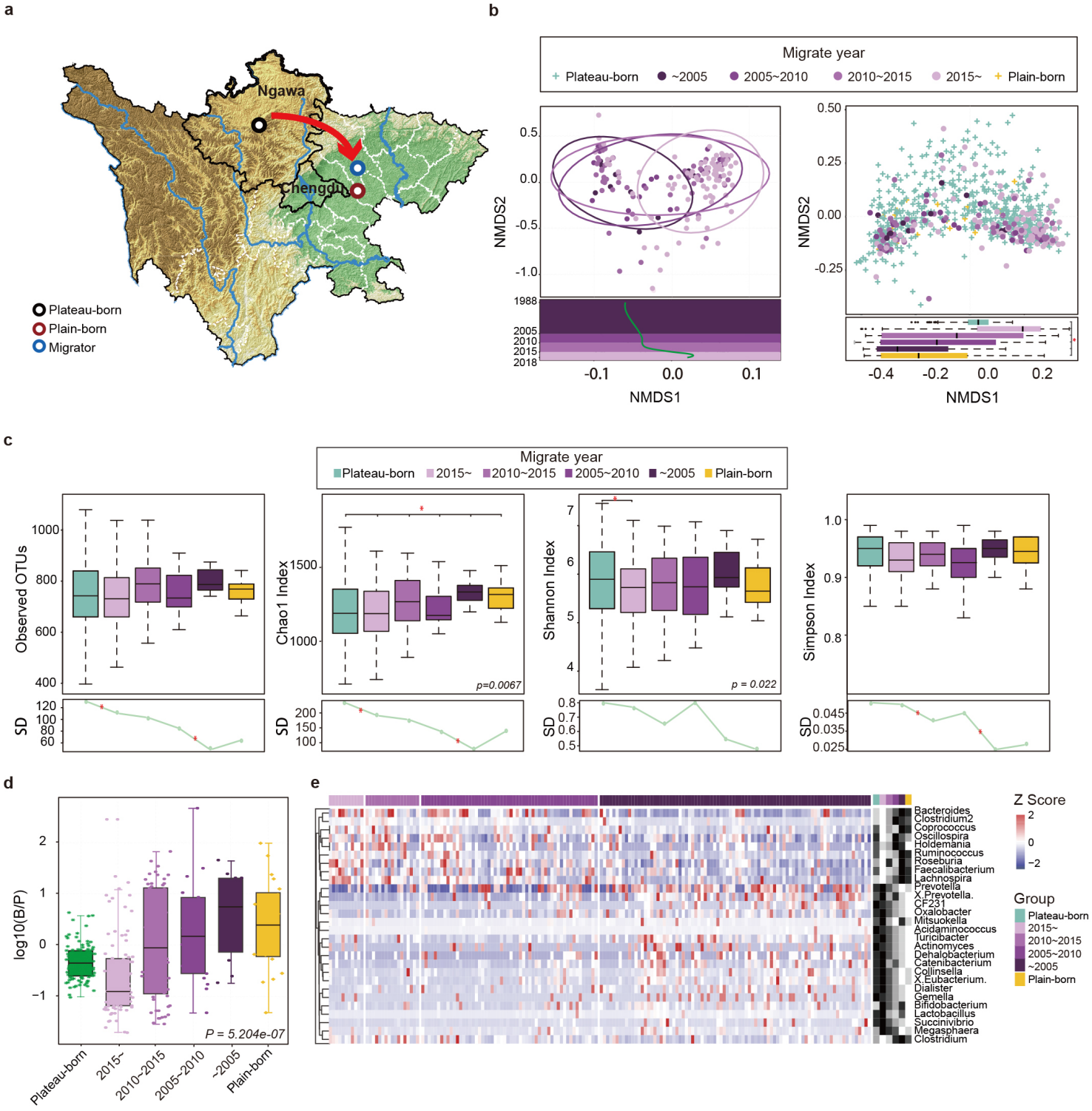

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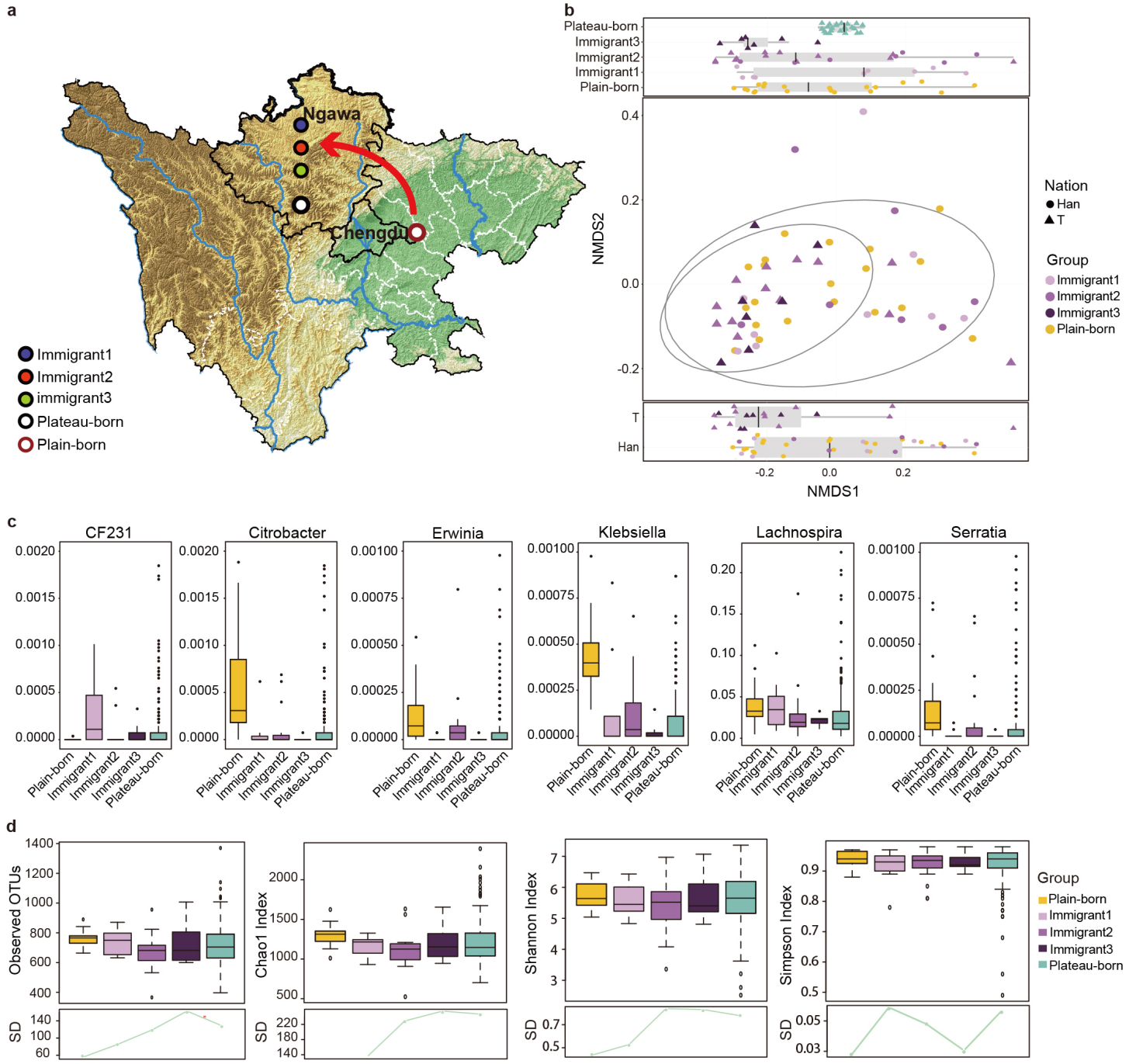

Figure 3

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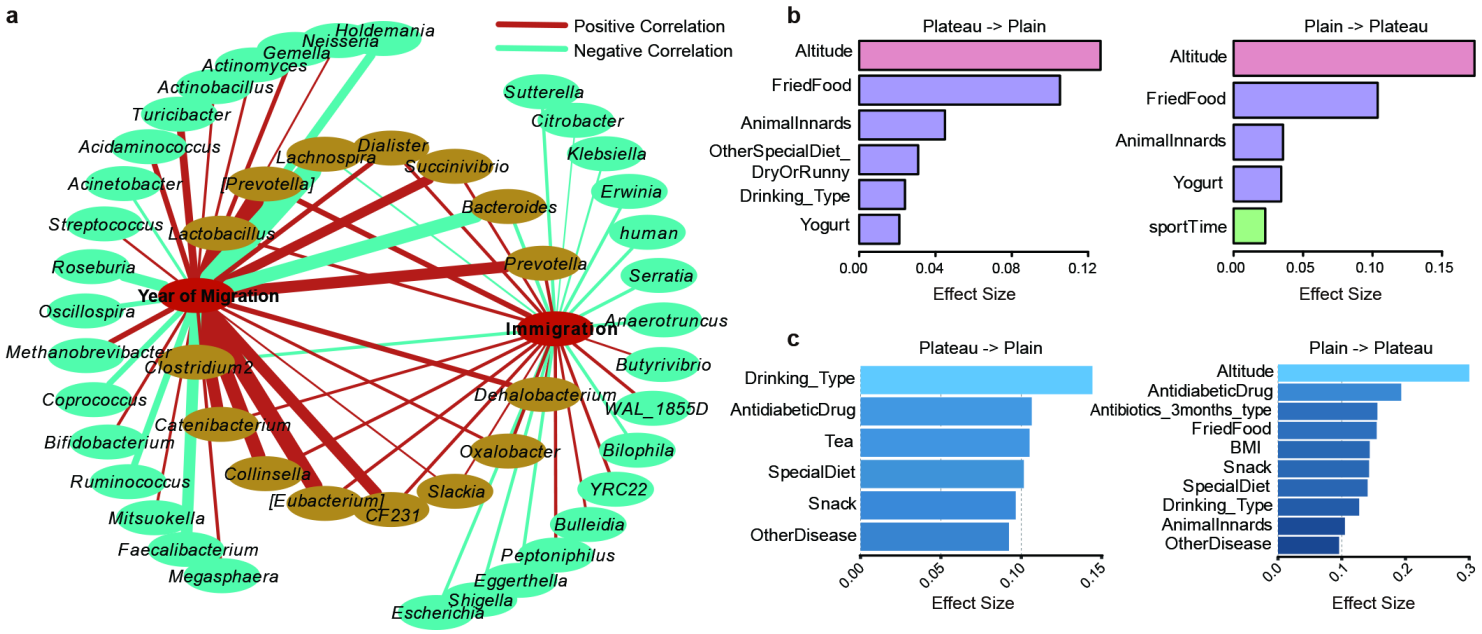

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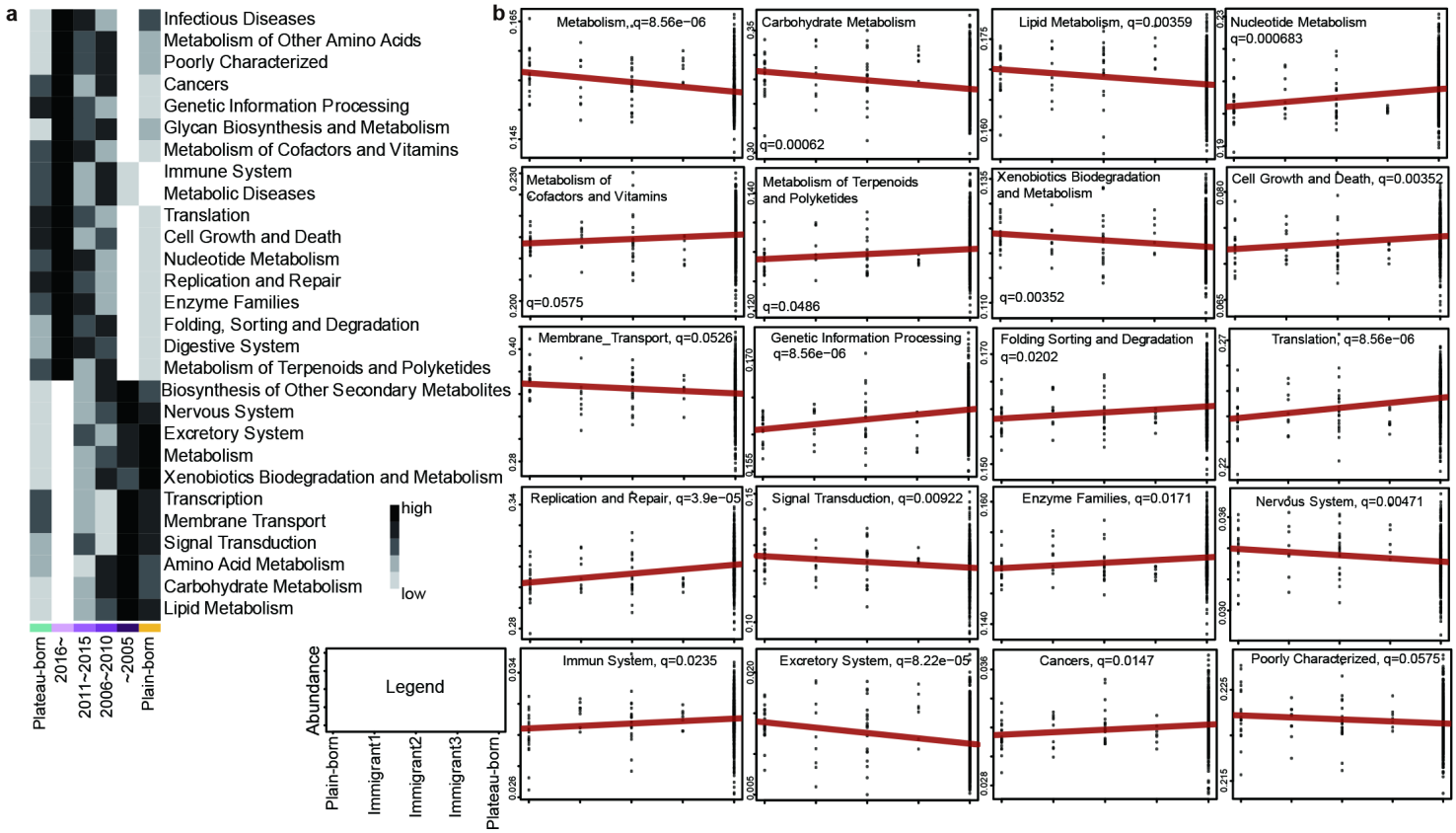

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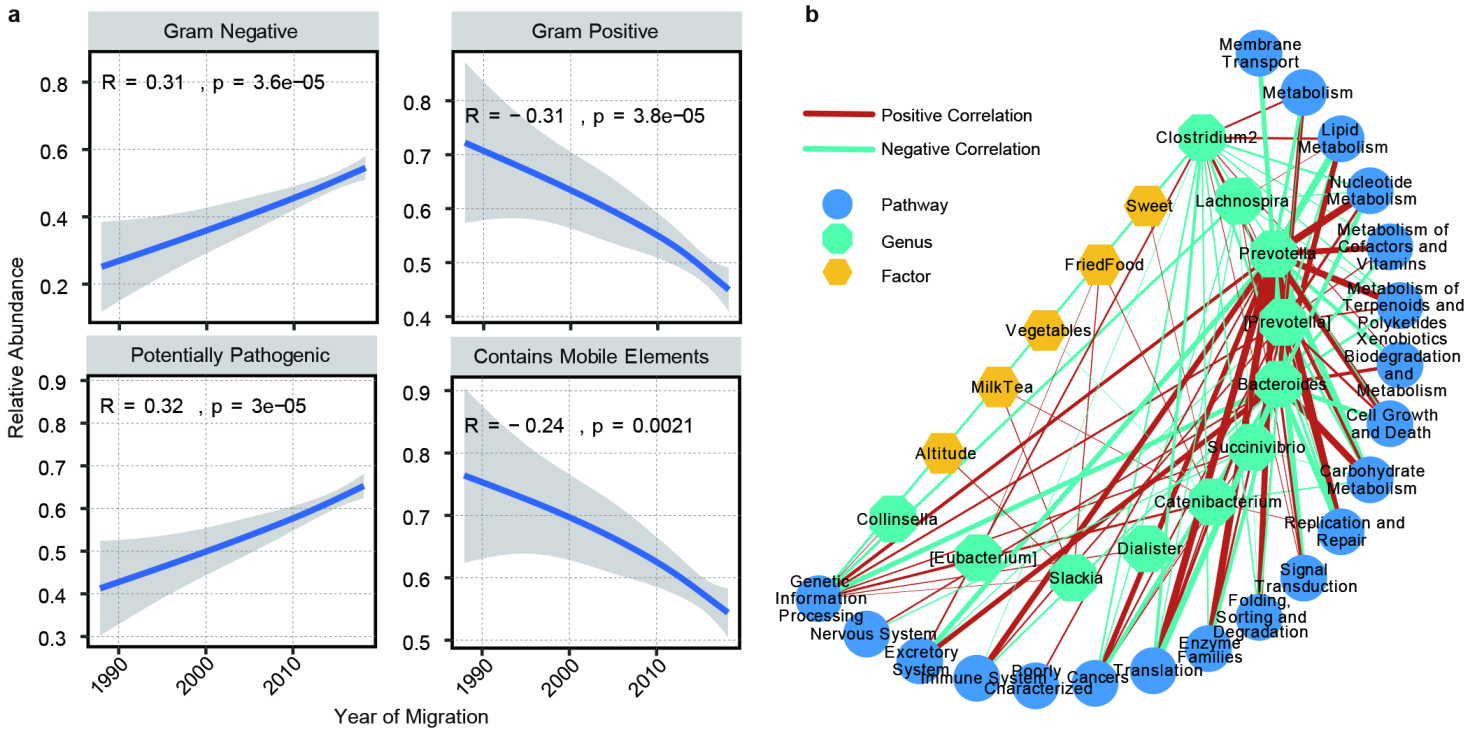

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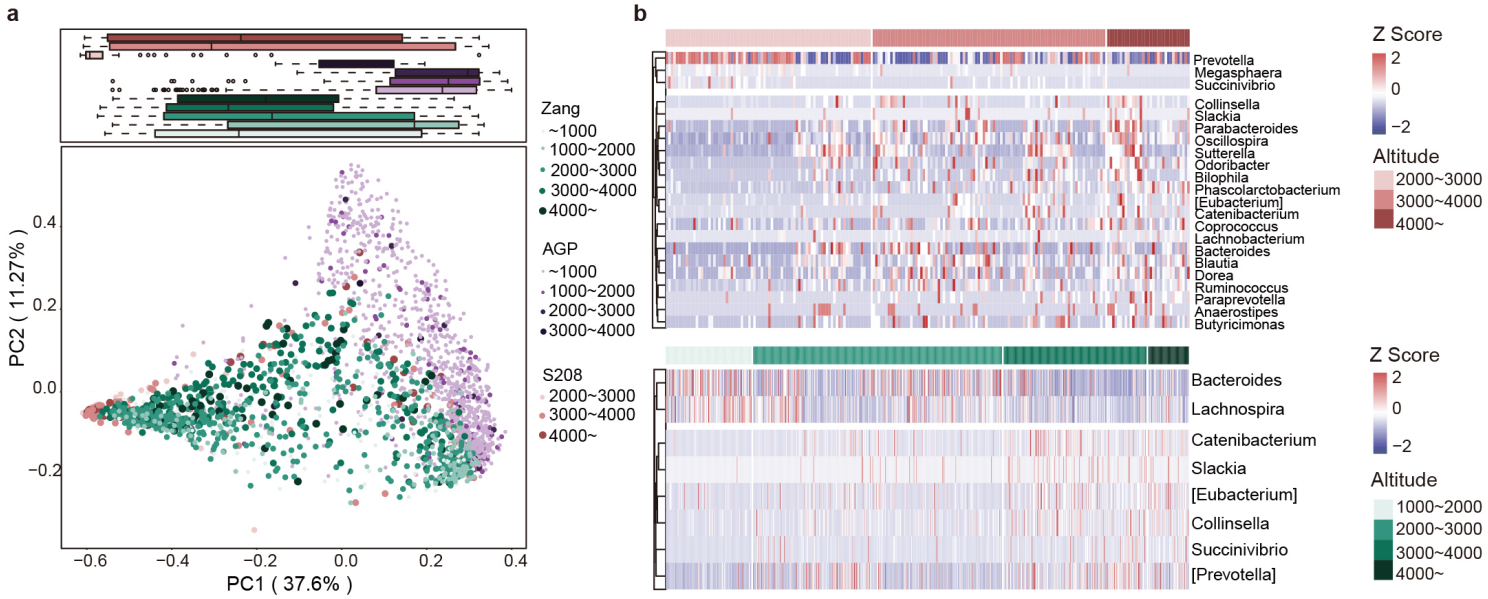

Figure 7

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