Bacterial community in saline farmland soil on the Tibetan Plateau:
Responding to salinization while resisting extreme environments

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Abstract

Background: Soil salinization caused by irrigation will reduce soil health and crop yields. Soil salinization has become one of the world's soil degradation problems. There are few studies on the response of microbial communities to soil salinization in plateau environments. Here, we applied metagenomics technology to make an exploration on the salinized soil microorganisms of the Tibetan Plateau.

Results: The metagenomic data results show that the microbial species diversity and genome diversity of saline soil and non-saline soil have changed significantly. We found that the abundances of chemoautotrophic and acidophilic bacteria comprising *Rhodanobacter*, *Acidobacterium*, *Candidatus* Nitrosotalea, and *Candidatus* Koribacter were significantly higher in saline soil. and the potential degradation of organic carbon in saline soil. The potential degradation of organic carbon in the saline soil, as well as the production of NO and N$_2$O via denitrification, and the production of sulfate by sulfur oxidation were significantly higher compared with the non-saline soil. Both types of soils were rich in genes encoding resistance to environmental stresses (i.e., cold, ultraviolet light, and hypoxia). The resistance of the soil microbial communities to the saline environment on the Tibetan Plateau is based on the absorption of K$^+$ as the main mechanism, with cross-protection proteins and absorption buffer molecules as auxiliary mechanisms. Network analysis showed that functional group comprising chemoautotrophic and acidophilic bacteria had significant positive correlations with electrical conductivity and total sulfur, and
significant negative correlations with the total organic carbon, pH, and available nitrogen. The soil moisture, pH, and electrical conductivity are likely to affect the bacterial carbon, nitrogen, and sulfur cycles.

**Conclusions:** These results indicate that the specific environment of the Tibetan Plateau and salinization jointly shape the structure and function of the soil bacterial community, and that the bacterial communities respond to complex and harsh living conditions. In addition, environmental feedback probably exacerbates greenhouse gas emissions and accelerates the reduction in the soil pH. This study will provide insights into the microbial response to soil salinization and the potential ecological risks for the special plateau environment.

**Keywords:** Saline, Tibetan Plateau, Metagenomics, Microbial community, Resistance mechanism

**Background**

The Tibetan Plateau is located at a high altitude (average > 4,500 m), with severe cold and low oxygen levels, and it is strongly affected by ultraviolet radiation [1, 2]. Due to global warming, population increases, and the fragility of the Tibetan Plateau environment, various ecological and environmental problems have occurred, such as vegetation degradation, biodiversity decline, desertification, and salinization [3, 4]. Soil is the basis of the function of the global terrestrial ecosystem [5] and soil salinization is considered one of the most pressing environmental challenges for the
The continued salinization of scarce agricultural soil resources will have feedback effects on global climate change, as well as detrimentally affecting the already poor living conditions for people on the Tibetan Plateau. However, the problem of saline soil and its environmental impact in the content of extreme climate change in this complex and fragile environment have received little attention.

Soil microorganisms are essential components of the soil ecosystem on the Tibetan Plateau and they play key roles in the health of the ecosystem [8, 9]. Soil microorganisms are involved in the conversion of most nutrients in the soil, and they have critical roles in the decomposition and stabilization of soil organic matter and the nutrient cycle, thereby influencing plant growth and the productivity of aboveground plants [10, 11]. High salinity has adverse effects on biological activities. The microbial community will adapt to changes in salinity by adjusting its composition and enhancing interactions [12, 13]. Microorganisms adapt to high salinity environment mainly through two mechanisms comprising the synthesis or absorption of organic osmotic agents, and absorbing K⁺ and other inorganic ions to resist osmotic stress [14, 15], thereby maintaining the normal life activities of cells under high osmotic pressure conditions. Soil samples from different high salinity regions vary greatly in microbial community structures, and bacteria are more sensitive than fungi [16, 17]. In-depth investigations of changes in the structure and function of bacterial communities as sensitive factors will help us to understand the mechanisms responsible for maintaining the function of saline soil ecosystems.
Studies of saline soils throughout the world have shown that salinity has important effects on the microbial community composition and metabolic functions. Salinity leads to significant decreases in the soil microbial diversity and biomass, reductions in the soil enzyme activities [18, 19], inhibition of bacterial growth and respiration [20], retardation of the organic matter degradation rate and suppression of nitrification [21]. The mechanisms of bacteria resisting high salinity environments consume large amounts of energy, and the organic matter in the soil will be consumed rapidly [22]. Bacteria with autotrophic capacities have survival advantages in a nutrient-poor environment, thereby leading to changes in the metabolic functional network for the bacterial community. However, no bacteria are specifically adapted to high-salinity soil environments and it is not easy to find bacterial indicator in salinity soil [23]. The soil microbial community on the Tibetan Plateau has responded to extreme environmental pressures via a unique metabolic mechanism [24, 25]. However, the microbial communities in saline soils at high altitude have not been investigated.

In the 6\textsuperscript{th} century BC, humans mainly settled in the northeast area of the Tibetan Plateau, and they did not extend their agricultural activities to the land higher than 3600 meters above sea level in the central and southern Tibetan Plateau until 3500 cal yr B.P [26]. The melting of glaciers and repeated freezing–thawing of permanently frozen soils caused by global warming have partially exposed the glacier-covered mineralized rock layers on the surface of the Tibetan Plateau. In addition, the
increased water yield has accelerated the leaching of various minerals in the rock and acid rock drainage into the rivers [27, 28]. Thus, irrigation using river water has resulted in large amounts of sulfate and metal ions being applied to land, leading to salinization of the soil in the study area. The environmental challenges encountered by soil bacterial communities in farmland in the study area include high soil salinity, temperature differences between the day and night, extremely strong ultraviolet radiation, limited oxygen, and other extreme conditions. Thus, bacterial survival under these conditions evolved specific survival strategies. In this study, we will focus on the: (1) characteristics of bacterial community in saline soil on the Tibetan Plateau, and their biogeochemical cycling processes, (2) the mechanisms associated with the responses to multiple environmental pressures, and (3) the potential impacts of bacterial communities in salinized soil on environmental climate.

**Methods**

**Soil sampling**

The study area is located in Naidong County, Shannan City, Tibet, with an average altitude of 3560 m. Yala Snow Mountain is a natural snow mountain glacier with the highest altitude in the area of 6647 m and it is the main water source. The study area has a temperate monsoon plateau climate and the air is thin. The average annual temperature in Naidong County is 8.8°C, the average annual pressure is 660.4 hPa,
the average annual solar radiation is 6018.9 MJ, and the average annual precipitation is 383.2 mm.

In May 2019, saline soil samples (SA) were collected from farmland near the Zhiqu River that had been planted with barley (Fig. S1), and nonsaline soil samples (CK) were collected as a control from farmland near the Yalong River (Xiangqu) that had also been planted with barley. Five subsamples were collected at each sampling site according to the four corners of a square and the center point, where the side length was about 10 m. The surface 5–20 cm soil layer was collected and each sample was packed in a 50-ml sterile centrifuge tube. The sample tubes were refrigerated with ice packs and returned to the laboratory within 24 hours. Each of the subsamples was passed through a 2-mm sieve in the laboratory to remove any stones and plant debris. The five soil subsamples from the same location were mixed to obtain one sample. The mixed saline soil samples were designated as S1, S2, S3, S4, S5, and S6, and the nonsaline soil samples as N1, N2, N3, N4, N5, and N6. Each soil sample was divided into two parts, and one was stored at 4°C for subsequent chemical tests and experiments in the laboratory. The other was kept at –20°C for DNA extraction.

**Geochemical analysis**

The soil samples were dried at 55°C and crushed, before passing through a 2-mm sieve. The soil samples were mixed at a soil: water ratio of 1:5 (w/v), shaken well, and allowed to stand for 48 h. The supernatant was passed through a filter membrane
with a pore size of 0.45 µm to prepare the test solution for the experiments. The soil electrical conductivity (EC) value was measured in a suspension with a soil:water ratio of 1:5 (w/v) using a CLEAN Conductivity Tester (CON30; FC Corporation, California, USA). The soil pH value was measured in a suspension with a soil:water ratio of 1:5 (w/v) using a pH meter (PB-10; Sartorius, Goettingen, Germany). The pore-water dissolved nitrate (NO$_3^-$) and sulfate (SO$_4^{2-}$) contents were analyzed by ion chromatography (DX-120, DIONEX, Bannockburn, IL, USA) [29]. The Total organic carbon (TOC) content (Calculated as carbon dioxide) was confirmed by using a high-frequency infrared Carbon-Sulfur Analyzer (LECO CS744, LECO Corporation, USA). Total nitrogen (TN) was analyzed using a Eurovector elemental analyzer (Isoprime-EuroEA 3000, Milan, Italy). The available nitrogen (AN) contents were determined with the alkaline digestion diffusion method. The total sulfur (TS) contents were measured using the infrared absorption method after high frequency combustion (High-speed Analyzer HWF-900A, Wuxi, China). Other trace metal(loid)s were analyzed by ICP-MS (ThermoFisher X-series, Franklin, MA, USA) and ICP-AES (TJA IRIS-Advantage, Franklin, MA, USA).

**DNA extraction, library construction, and metagenomic sequencing**

The total DNA was extracted from each soil sample (0.5 g) using a PowerSoil DNA Extraction Kit (MoBio Laboratories, Carlsbad, CA, USA). The quantity and quality of
isolated DNA were evaluated using a NanoDrop spectrophotometer (ND-2000, Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis (Bio-Rad, Hercules, CA, USA), respectively. The extracted DNA was stored at –20°C until further analysis, or at –80 °C for long-term storage.

DNA was fragmented to an average size of about 300 bp using Covaris M220 (Gene Company Limited, China) for paired-end library construction. Paired-end library was prepared by using TruSeq™ DNA Sample Prep Kit (Illumina, San Diego, CA, USA). Adapters containing the full complement of sequencing primer hybridization sites were ligated to the Blunt-end fragments. Paired-end sequencing was performed on Illumina HiSeq3000 platform (Illumina Inc., San Diego, CA, USA) at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) using HiSeq 3000 PE Cluster Kit and HiSeq 3000 SBS Kits according to the manufacturer’s instructions (www.illumina.com)

**Bioinformatics**

The 3’ and 5’ ends were stripped (https://github.com/jstjohn/SeqPrep) and low-quality reads were removed (https://github.com/najoshi/sickle). The software SOAPdenovo (http://soap.genomics.org.cn, Version 1.06) was employed to assemble short reads and K-mers were tested for each sample. The software Scaffolds was employed to gene prediction and annotation after with a length over 300 bp were extracted and broken into contigs without gaps. The software CD-HIT
(http://www.bioinformatics.org/cd-hit/) was employed to all sequences sequence identity (90% coverage) from gene sets with ≥ 95%, and were clustered as the non-redundant gene catalog using. After quality control, the software SOAPaligner (http://soap.genomics.org.cn/) was employed to mapped reads to representative genes with ≥ 95% identity, and the gene abundances were evaluated in each sample. The software BLASTP (Version 2.2.28+, http://blast.ncbi.nlm.nih.gov/Blast.cgi) was employed to taxonomic annotations by aligning non-redundant gene catalogs against NCBI NR database with cutoff: 1e-5 (e-value). The software BLASTP (Version 2.2.28+) was employed to annotation the KEGG pathway search against the Kyoto Encyclopedia of Genes and Genomes database (http://www.genome.jp/kegg/) with an cutoff: 1e-5 (e-value).

**Statistical analyses**

Trimmomatic software was used to excise primers and for quality filtering with the original metagenomic sequences [30]. MetaPhlan2 software was then used to analyze the data and obtain the classifications for the microbial population with a degree of horizontal precision [31]. The species concentration in each sample type was calculated by comparing the mean and median relative abundances in the saline and nonsaline soil samples. R was used to conduct statistical analyses and to plot the taxonomic information at the genus level. The Shannon diversity index was calculated
using the “vegan” package [32]. The Bray–Curtis dissimilarity between different
sample types was calculated using the R package “ecodist” [33].

KEGG Orthology (KO) functional profiling of the soil microbiota was performed
using assemblies derived from whole-genome shotgun sequencing data. Low-quality
reads were first trimmed from raw sequencing data using Trimmomatic. High-quality
reads were assembled de novo into contigs using metaSPAdes [34] with the default
parameters. Next, we performed gene prediction for these scaffolds using PROKKA
V.1.11 [35] and the predicted proteins were assigned to the KO using the Kyoto
Encyclopedia of Genes and Genomes (KEGG) Automatic Annotation Server.
Trimmed high-quality reads located on the given scaffolds were counted to calculate
the abundances of Kos in each sample using the Burrows–Wheeler Aligner [36]. The
matrix was normalized by dividing the absolute amount of each functional gene by
the total number of reads assigned to functional genes in each sample in order to
determine the differential expression of microbial functional pathways in the saline
and nonsaline soil samples.

The bacterial correlations in the saline and nonsaline soil samples were computed
based on the relative abundance of each genus using SparCC with 100 bootstraps to
estimate the $p$-values for co-occurrence network analysis. The correlation values $p <
0.05$ were retained. The co-occurrence network obtained for the microbial
communities in the saline and nonsaline soil samples was visualized with Gephi
Community clustering was conducted based on the Bray–Curtis distance for principal coordinate analysis at the genus level, and ADONIS (“vegan”) analysis was performed to assess the similarities between groups and the significance of the differences between groups. Genera variation analysis to contrast the two soil sample types was conducted using the Wilcoxon rank sum test with R. Checks and corrections of the false discovery rate (FDR) were performed using the R program fdrtool.

Results and Discussion

Soil characteristics of Tibetan Plateau

In figure 2, soil geochemical analysis showed that the EC values determined for the saline soil were about 9 ds • m⁻¹, and thus the soils were moderately saline [37]. The EC values of the nonsaline soil were less than 4 ds • m⁻¹. The pH values were ~4.5 for the saline soil and ~7.2 for the nonsaline soil. TOC of saline and nonsaline soil were about 1.2% and about 4.1% respectively. The soil moisture contents were about 7% higher in nonsaline soil than the saline soil, possibly because salinization destroyed the physical structure of the soil. The nitrate, sulfate, and TS accumulations were significantly higher in the saline soil than the nonsaline soil, whereas TN and AN were significantly lower in the saline soil. Thus, the saline soil was acidic and the
nutrient nitrogen contents were lower. The long-term accumulation of heavy metal(loid)s resulted in an extremely high Mn content. The levels of heavy metal(loid)s such as Zn, As, Cu, and Cr, and metal cations such as K\(^+\), Ga\(^{2+}\), and Mg\(^{2+}\) were also significantly higher in saline soil than nonsaline soil (Fig. S2). In general, the saline and nonsaline soil differed significantly in terms of most of the geochemical parameters (FDR < 0.05, Fig S2). The saline soil had a low pH, high salinity, and low nutrient levels, and it was also affected by the extreme unique climate of the Tibetan Plateau, including cold, hypoxia, and strong ultraviolet radiation. Microorganisms may produce a series of community changes and genetic selection under such environmental conditions.

**Bacterial community in saline and nonsaline soil of Tibetan Plateau**

After removing low-quality reads of metagenomic sequencing, the quality control results (Table S1) and predicted open reading frames after assembly (Table S2) showed that the average Shannon index was 5.43 for saline soil and that for nonsaline soil was 5.35 (Table S3). In all samples, bacteria accounted for approximately 98.07% of the total sequences, archaea accounted for approximately 1.32%, and fungi accounted for only 0.01%. The microbial community was dominated by bacteria, and the proportions of archaea and fungi were extremely low. At the microbial phylum
level, the sequences were dominated by Proteobacteria, Actinobacteria, Acidobacteria, Gemmatimonadetes, Chloroflexi, and other phyla (Fig. 1a).

Figure 1. Composition and differences in saline and nonsaline soil on the Tibetan Plateau. a. Bacterial composition at the phylum level in saline and nonsaline soil samples of Tibetan Plateau. b. Top 20 genera with significant differences (FDR < 0.05) in saline (orange) and nonsaline (cyan) soil samples of Tibetan Plateau, the total number of reads is normalized to 100000. c. Principal coordinate analysis of saline (orange) and nonsaline (cyan) soil samples based on the composition and abundances of the bacterial communities at the genus level.
By analyzing the differences in the bacterial compositions in two types of soil samples (Wilcoxon’s test, FDR < 0.05, Fig. 1b), we found that the significantly enriched bacteria in the saline soil had different metabolic strategies. Most were heterotrophic bacteria, but some were chemoautotrophs, such as *Rhodanobacter*, *Granulicella*, and *Acidobacterium*. The dominant significantly enriched bacterial groups associated in the carbon and nitrogen cycles identified in nonsaline soil. For example, *Microvirga* and *Hyphomicrobium* have denitrification functions [38, 39], and *Candidatus Nitrosocosmicus* has ammonia oxidation and carbon fixation capacities [40]. The bacteria in saline soil were found to have specific environmental adaptations (such as chemoautotrophs and obligate acidophilic), whereas the dominant bacteria in nonsaline soil have greater capacity for carbon and nitrogen assimilation. PCoA showed that all of the saline soil samples clustered together and those in nonsaline soil samples formed another cluster, thereby indicating that soil salinization led to significant differences in the bacterial community structure (Fig. 1c).

The results of the functional abundance based on the KEGG database (Fig. S3a) showed that significant differences in functional genes related to environmental stress resistance (ultraviolet radiation resistance, temperature change response, oxygen limitation response, and salinity adaptation) and important biogeochemical processes (e.g., carbon fixation, nitrogen metabolism, sulfur metabolism, methane metabolism, and heavy metal resistance). PCoA based on the functional composition showed (Fig.
S3b and c) that two separate clusters were formed at both module and KO levels, indicating that salinization also led to significant differences in the soil bacterial functions (ADONIS, p-value < 0.05). However, the certain bacterial metabolism related to different element cycles and environmental stress responses should be revealed deeply.

**Metabolic pathways in saline and nonsaline soil samples of Tibetan Plateau**

**Carbon cycling**

The carbon cycle mainly comprises carbon fixation, carbon degradation, and methane metabolism, which is important for microorganisms in the soil to obtain energy and materials[41]. The carbon fixation pathways (Fig. S4a) in all the samples were mainly composed of the reductive citrate (rTCA) cycle, hydroxypropionate hydroxybutylate (3-HP/4-HB) cycle, crassulacean acid metabolism (CAM) pathway, and Wood–Ljungdahl (WL) pathway. In Fig. 2, only the abundances of the *ackA* and *folD* genes were significantly higher in saline soil than nonsaline soil, and these genes are involved in the 3-HP/4-HB cycle and WL pathway, respectively. Thus, the genes such as *korA*, *sdhA* and *ppdK* with significantly higher abundances in saline soil participated in the 3-HP/4-HB cycle and WL pathway, whereas the genes with significantly higher abundances in nonsaline soil mainly participated in the rTCA cycle. The rTCA cycle is prevalent in anaerobic bacteria that are adapted to hypoxic
environments on the Tibetan Plateau and this cycle only requires two ATP equivalents to form pyruvate [42]. The abundance of the WL pathway was probably higher because of its extremely low energy consumption (requirement < 1 ATP) and requirement for strict anoxic conditions [43]. Thus, low nutritional availability and extreme environments explain why the rTCA cycle and WL pathway predominate in the soil on the Tibetan Plateau, where the 3-HP/4-HB cycle pathway are adaptations to the nutritional deficiencies, respectively.

\[
y = -0.0287 + 1.09x \quad R^2_{adj} = 0.97 \quad p < 0.001
\]

\[
y = -0.437 + 1.01x \quad R^2_{adj} = 0.9 \quad p < 0.001
\]

\[
y = -0.0118 + 1.05x \quad R^2_{adj} = 0.96 \quad p < 0.001
\]

* FDR < 0.05

- Carbon Fixation
- Carbon Degradation
- Methane Metabolism
Figure 2. The ratio of genes in carbon cycling pathway in saline and nonsaline soil on the Tibetan Plateau. The black dotted line equation “y = x” indicates that the horizontal and vertical axes are equal. Green, red, and blue represent genes of carbon fixation, carbon degradation, and methane metabolism pathways, respectively. Genes with significant differences (FDR < 0.05) are marked in the corresponding colors and connected with short lines. The total number of reads is normalized to 100000.

In the carbon degradation pathway (Fig. 2), the genes with significantly higher abundances in saline soil included genes related to starch degradation comprising *cdd* and *SGAI*, chitin degradation gene *NAGLU*, lignin degradation gene *yfiH*, cellulose degradation gene *bcsZ*, hemicellulose degradation gene *xynC*, and *rexA*, indicating that the potential for carbon degradation was greater in the saline soil. Interestingly, the abundances of genes associated with the degradation of labile carbon (starch, pectin, and hemicellulose) and recalcitrant carbon (cellulose, chitin, and lignin) were higher in the saline soil, possibly because these mechanisms allow microorganisms to maintain their ecosystem functions in the short term in saline soil. In addition, these mechanisms may also explain why the TOC contents were significantly lower in saline soil than nonsaline soil, and the collapse of farmland ecosystems may occur if saline soil remains oligotrophic for a long time [24, 25]. There were no significant differences in the abundances of genes related to methane metabolism in the two soil types (Fig. 2 and Fig. S4b). The lack of significant differences in abundances of genes
related to this process indicates that salinization of the soil had no significant impacts on methane metabolism.

Nitrogen cycling

The nitrogen cycle is one of the crucial soil nutrient cycle processes for the growth of crops and it is driven by soil microorganisms with specific functions [44]. The abundances of genes related to dissimilatory nitrate reduction and denitrification in the soil microbial nitrogen cycle differed significantly in saline and nonsaline soil (Fig. 3), but they did not differ significantly in the nitrate assimilation reduction pathway.
Figure 3. Differences in the abundance of genes related to nitrogen cycling in the saline (orange) and nonsaline (cyan) soil on the Tibetan Plateau. Bar plots show the normalized abundances of nitrogen cycling genes. Significantly different (FDR < 0.05) genes are marked with “*” and circled numbers. Undetected genes are indicated in gray. Circled numbers identify genes with significant differences: 1, dissimilatory nitrate reduction; 2, nitrification; 3, denitrification; and 4, organic nitrogen conversion. The total number of reads is normalized to 100000.
In the denitrification pathway, the abundance of gene *nirK* (nitrite reductase catalyzing N$_2$O to NO) was significantly higher in saline soil (FDR < 0.05) than nonsaline soil. The presence of higher amounts of NO is likely to proceed forward to produce more N$_2$O. In saline soil, bacteria have the potential to produce more NO and N$_2$O via denitrification, and the abundances of genes *nosZ* that could reduce N$_2$O were lower in saline soil than nonsaline soil, which were resulted in more NO and N$_2$O produced in saline soil. The abundances of the dissimilatory nitrate reduction genes *napA* and *nrfA* were significantly higher (FDR < 0.05) in saline soil than nonsaline soil (Fig. 3), demonstrating that the potential for ammonia conversion was higher in nonsaline soil. Significantly higher abundances were found in nonsaline soil of the gene *gdhA* encoding the enzyme that catalyzes the conversion of ammonia to L-glutamate, which are all involved in the conversion of ammonia to glutamic acid (FDR < 0.05). It is indicated that more ammonia could be converted into organic nitrogen and this is beneficial for the production of crops. Microorganisms need to synthesize large amounts of amino acids to resist environmental pressure [45]. Therefore, the input of ammonia is critical for the microbial community.

**Sulfur metabolism**

Due to the high input of sulfate in saline soil, we analyzed the differences in the genes abundance of three pathways related to sulfur metabolism (assimilatory sulfate reduction, dissimilatory sulfate reduction and oxidation, and SOX system) (Fig. 4).
The abundances of genes related to environmental sulfide absorption were significantly lower in saline soil, but the abundances of genes associated with the elimination of toxic intracellular sulfide were significantly higher.

**Figure. 4. Difference in abundances of sulfur cycling genes in saline (orange) and nonsaline (cyan) soil on the Tibetan Plateau.** The total number of reads is normalized to 100000.
First, in saline and nonsaline soil, the abundance was high for the assimilatory sulfate reduction module that consumes sulfate in the environment and ultimately synthesizes sulfur-containing amino acids. This pathway was probably dominant because of the extremely high sulfate content in saline soil and the large demand for amino acids in the bacterial community. Second, in both soil types, the abundance was low for the dissimilatory sulfate reduction and oxidation module that produces energy and inorganic sulfides, and the abundance of dsrAB (dissimilatory sulfite reductase) was extremely low, probably because “reverse” sulfite reductase (dsr) is not necessary for the oxidation of sulfide or thiosulfate, and sat (encoding the enzyme that catalyzes the conversion of sulfate to Adenylyl sulfate) and aprAB (adenylylsulfate reductase) participate in the energy production process but they produce cytotoxic sulfites, so their abundances were also low. Finally, the abundance of the soeBC gene encoding the enzyme in the SOX system that catalyzes the conversion of sulfite into sulfate was significantly higher in saline soil, and this enzyme is important for sulfite oxidation in the cytoplasm (Fig. S5). The oxidation of sulfur can reduce the toxicity of sulfite in cells [46], as well as providing electrons and energy to cells [47].

**Metal resistance**

In this study, the abundances of heavy metal(loid) resistance genes such as copB, cutC, cusRS, and pcoB genes that confer tolerance to copper and the manganese transport
gene *mntH* were significantly higher in saline soil (Fig. 5). The differences in the functional genes related to heavy metal absorption showed that the gene *arsB* related to arsenic absorption had a significantly higher abundance in saline soil (Fig. 5). Both soil types had high abundances of the arsenate reductase gene (*arsC*), but the abundance of the arsenite oxidase gene (*aoxAB*) was extremely low because the hypoxic environment promoted the migration of arsenic [48]. The accumulation of arsenite in saline soil on the Tibetan Plateau has toxic effects on microorganisms and crops. However, the abundance of gene *arsH* (arsenical resistance protein) was extremely low in both soil types (Fig. S6), and thus, the soil bacteria did not have a high capacity to resist the accumulation of arsenic in study area.
Figure 5. Abundance of heavy metal(loid) resistance genes in saline and nonsaline soil on the Tibetan Plateau. The black dotted line with the equation “y = x” indicates that the horizontal and vertical axes are equal. Genes with significantly different abundances (FDR < 0.05) are marked in purple and connected with short lines. The total number of reads is normalized to 100000.

These results indicate that two main mechanisms mediate microbial resistance to the toxicity of heavy metal(loid)s in saline soils on the Tibetan Plateau, i.e., the efflux of excessive concentrations of heavy metal(loid) ions from cells and expressing proteins that confer tolerance of heavy metal(loid) ions. However, bacteria to resist
increasing concentrations of heavy metal(loid)s is possibly not sufficient for increasing concentration of heavy metal(loid) ions in the soil of Tibetan Plateau, which should be studied in future research.

**Environmental stress response**

![Figure 6. Abundance of the environmental stress resistance genes. The total number of reads is normalized to 100000.](image)

Ultraviolet radiation resistance, the nucleotide excision repair pathway, and photoreactivation (DNA repair via the photolysis enzyme encoded by the *phrB* gene)
are applied by bacteria to avoid and repair damage due to ultraviolet radiation [49] (Fig. 6). The abundances of the recNO and alkB genes associated with DNA repair were significantly higher in saline soil due to more DNA damage in saline soil (Fig. S8). Bacteria adapt to cold environments mainly through cold shock genes cspA, desK, and desR, which encode enzymes that protect cells from ice crystal damage, and that maintain the transcription and translation processes within cells [50, 51], and via lipid desaturases (desA1, desA2, and desC; Fig. S7) and the synergy among unsaturated fatty acid synthesis genes fabAB (anaerobic) and desAB (aerobic), which are responsible for synthesizing short-chain unsaturated fatty acids embedded in the cell membrane to maintain the cell membrane fluidity and avoid film hardening at low temperatures [52, 53]. These genes were abundant in the two soil types and the abundance of fabAB was higher than that of desAB due to low oxygen levels on Tibetan Plateau (Fig. S7). Bacteria express large amounts of catalase and peroxidase when responding to oxygen limitation stress [40]. Thus, the abundances of the cydB, fnr, and oxyR genes were significantly higher in saline soil (Fig. S7), whereas the katE (catalase) gene was more abundant in nonsaline soil, and the enzyme encoded by katE also acts as a cross-protection protein to help cells cope with environmental pressures. The response mechanisms to oxygen limitation differed between the two soil types. Molecular chaperones help protein folding and refolding to enhance stress resistance [54], and many of these genes had high abundances in the two sample types (Fig. S7). However, the abundances of danK and groEL were significantly (FDR < 0.05) higher
in CK, whereas grpE and pccA were more abundant in SA, and the abundances of major molecular chaperone genes were higher in nonsaline soil than saline soil (Fig. S7), which lacked sufficient energy and substrates to synthesize the required molecular chaperones.

In response to high salinity and low pH, the abundance of the K⁺ high-affinity transport system (kdpABC) was significantly higher in saline soil (Fig. S7). Bacteria generate an inward positive membrane potential through the active inflow of K⁺ to partially deflect the inward flow of protons [55], as well as helping cells to resist the stress due to high osmotic pressure. The metabolism of proton buffer molecules can also maintain the pH in the cytoplasm, and the abundance of the phosphate uptake gene pstS was significantly higher in saline soil (Fig. S7). Cross-protective genes encoding proteins (osmC, dps, and katE) that maintain the normal life activities of cells under high osmotic pressure were abundant in saline soil, where the abundance of osmC was significantly higher. In addition, the microbial self-synthesizing glycine betaine gene gbsAB was more abundant in nonsaline soil, whereas proline and glycine betaine absorption genes (opuABCD, proP, putABP, etc.) were more abundant in saline soil (Fig. S7), probably because the energy consumed by the synthetic permeate was higher than that absorbed from the environment. Therefore, the soil bacteria in saline soil were deficient in substances and energy, so they employed low energy consumption mechanisms to absorb K⁺, phosphate, and osmotic substances from the
environment, as well as synthesizing a small amount of protective proteins to resist the low pH and high osmotic pressure stresses.

Thus, the bacterial community in saline soil was affected by more extreme environmental stresses than that in nonsaline soil. Low pH and high osmotic pressure resistance genes were more abundant, and the abundances of molecular chaperone genes were significantly lower. Genes related to adaptability to the specific climatic conditions on the Tibetan Plateau were abundant and the differences in their abundances in the two soil types were not obvious.

**Effects of physicochemical parameters on microbial community and metabolic capacity**

Co-occurrence network analysis based on the bacterial genera, functional genes, and environmental factors was constructed in order to understand how environmental factors affect the complex bacterial community structure and functions in saline and nonsaline soils on the Tibetan Plateau (Figs. 7 and S8). The network based on the bacterial community and environmental factors contained three bacterial modules (Table S3). The main negative correlations were found between these three modules, but the bacterial genera within the same module were mainly positively correlated (Fig. 7).
Figure 7. Network of the top 100 dominant genera and physicochemical parameters. A connection denotes a Spearman’s correlation coefficient with a magnitude greater than 0.6 (positive correlation = red lines) or less than −0.6 (negative correlation = green lines) and statistically significant ($p$-value $< 0.05$). The size of each node is proportional to the number of connections (i.e., degree). The thickness of each connection between two nodes (i.e., edge) is proportional to the Spearman’s correlation coefficient (i.e., weight), ranging from $|0.6|$ to $|1|$. The network is colored according to the modules, where the nodes clustered in the same module share the same color. The lower right corner is a schematic diagram, and the large circles represent three modules based on network analysis, and the positions are
corresponding. In the large circles, the top number indicates the number of nodes, the red circle and the number indicate positive correlation within the module, and the green indicates negative correlation. Between the large circles, red lines and numbers indicate positive correlations, and green lines and numbers indicate negative correlations.

Module 0 (Orange) contained a total of 22 nodes and most nodes (> 50%) represented microbial genera with a significant (FDR < 0.05) higher abundance in saline soil. Most of the genera are chemoautotrophic and acidophilic bacteria (Table S5 and Fig. 1a), which have strongest correlations with the soil physicochemical parameters, including positive correlations with EC, sulfate, total sulfur, and nitrate, but mostly negative correlations with pH, TN, AN moisture, and TOC (Fig. 7). Module 1 (purple) contained 42 nodes. All of the genera are high abundant in the two soil types. These genera had strong positive correlations with the soil moisture, TOC, and AN, weak positive correlations with TN and pH, and mostly negative correlations with other environmental factors (Fig. 7). Module 2 (cyan) contained 36 nodes, and these genera were in saline and nonsaline soil. They had very weak negative correlations with TN and pH, and almost no correlations with other environmental factors. Overall, EC, AN, moisture, TOC, and pH were the most influential environmental factors. Bacterial community responded to the environmental factors by forming different functional groups (Fig. 7), indicating that the stress due to
salinity indeed altered the topological roles of microbes and reorganized the keystone populations [56].

The network constructed based on the functional genes and environmental factors in different metabolic pathways (Fig. S8 and Table S4) showed that the main physical and chemical parameters in saline soil (EC, pH, TOC, moisture, AN, TS, etc.) affected the key biogeochemical cycles for C, N, S, As, and other elements in the soil (Fig. S8). TOC was significantly positively correlated with rTCA cycling which was a high energy efficiency conversion rate pathway in the carbon cycle. However, TOC was significantly negatively correlated with the WL pathway, which may have been related to the extremely low energy consumption and strict anaerobic requirements of this pathway. In addition, TOC, moisture, and pH were significantly negatively correlated with the main carbon degradation genes (cdd, SGA1, rexA, xynC, bcsZ, and NAGLU), and the high abundance of carbon degradation genes resulted in a significant decrease in the soil TOC concentration (Fig. 3). EC was negatively correlated with carbon fixation genes and positively correlated with carbon degradation genes, indicating that high salinity led to the rapid degradation of organic carbon for the bacterial requirements for substrates and energy. Most of the genes in nitrogen cycle were also positively correlated with TN, AN moisture, and pH, but negatively correlated with EC (Fig. S8). In particular, the abundance of gene nirK was significantly negatively correlated with TN, but significantly positively correlated with pH, indicating that the denitrification process in saline soil was affected by
changes in the soil physicochemical properties, and the long-term accumulation of salinity may have led to the accumulation of nitrate and enhanced denitrification. In addition, sulfate and total sulfur were significantly negatively correlated with the sulfate absorption gene cysAPUW, but significantly positively correlated with the sulfur oxidation gene soeABC, while the key gene sat in the sulfate reduction pathway was significantly negatively correlated with EC. These results suggest that the accumulation of sulfide in saline have resulted in the bacteria producing more sulfur oxidation genes to synthesize more sulfate. The secondary accumulation of salt would happen in the soil. Moreover, the soil moisture and pH were negatively correlated with arsenic resistance genes, whereas EC had positive correlations. Overall, high salinity and the accompanying changes in the soil properties had significant impacts on the microbial community and its metabolic network, with significant increases in the chemoautotrophic and acidophilic bacterial modules, as well as effects on other heterotrophic bacteria modules related to the carbon and nitrogen cycles. The bacterial community in the saline soil is likely to consume more soil organic carbon to increase denitrification and intensify the oxidation of sulfur.

Conclusions

Global warming has caused the melting of glaciers and the repeated freezing and thawing of the permanently frozen soil on the Tibetan Plateau, resulting in a sudden increase in the water volume and the exposure of rock formations, where the water
flow has washed over these mineral-rich rock formations into rivers to subsequently increase the soil salinity via irrigation. In this study, we found that the salinization of the soil was accompanied by increased acidity and the accumulation of metal. People who live along the rivers on the Tibetan Plateau are affected by the risk of increasingly saline farming soil. Soil salinization significantly changed the bacterial communities on the Tibetan Plateau, and chemoautotrophic and acidophilic bacteria became dominant. In addition, the key biogeochemical cycle function clusters changed. Carbon degradation, denitrification, and sulfur oxidation gene clusters were highly abundant in saline soils, which were associated with the loss of soil organic matter, increased emissions of NO and N₂O, and higher sulfate levels in local area. The bacterial community adapting to saline soil did not alleviate the degree of soil salinity, and bacterial community is likely to consume more energy to cope with the extreme climate under saline soil conditions due to the unique features of the Tibetan Plateau. The Yalong River Basin in the study area is a major tributary in the middle reaches of the Yarlung Zangbo River and the most important agricultural area in Shannan City, Tibet. The continuous salinization of agricultural soil along this river will have severe detrimental effects on the local economy and environment. Soil salinization also becomes one of the most direct forms of feedback on the Tibetan Plateau in response to global climate change.
Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2017) in National Genomics Data Center (Nucleic Acids Res 2021), China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences, under accession number CRA003771 that are publicly accessible at (http://bigd.big.ac.cn/gsa/s/6AYB1I5n).

Competing interests

The authors declare that they have no competing interests.

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**Authors’ contributions**

YL, XG and LH were responsible for collection of samples. XW performed DNA extractions and preparation. LH, CQ and XL measured and analyzed the environmental factors. YL and YC performed sequence assembly, annotation, analysis. QL and YL visualized most of figures. XL and XG developed the project design and provided project oversight. Both YL, XW and XG contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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