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**Microbial community structure and diversity within
hypersaline Keke Salt Lake environments**

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Abstract

Keke Salt Lake is located in the Qaidamu Basin of China, and is a unique magnesium sulfate-subtype hypersaline lake that exhibits a halite domain ecosystem, yet its microbial diversity has remained unstudied. Here, the microbial community structure and diversity was investigated via high-throughput sequencing of the V3-V5 regions of 16S rRNA genes. A high diversity of OTUs were detected for Bacteria and Archaea (734 and 747, respectively) which comprised 21 phyla, 43 classes, and 201 genera of Bacteria and 4 phyla, 4 classes, and 39 genera of Archaea. Salt-saturated samples were dominated by the bacterial genera *Bacillus* (51.52%–58.35% relative abundance), *Lactococcus* (9.52%–10.51%) and *Oceanobacillus* (8.82%–9.88%) within the Firmicutes phylum (74.81–80.99%) contrasting with other hypersaline lakes. The dominant Archaea belonged to the Halobacteriaceae family, and in particular, the abundant genera (>10% of communities) *Halonotius*, *Halorubellus*, *Halapricum*, *Halorubrum* and *Natronomonas*. Additionally, we report the presence of Nanohaloarchaeota and Woesearchaeota in Qinghai-Tibet Plateau lakes, which has not been previously documented. Total salinity (especially Mg^{2+} , Cl^- , Na^+ and K^+) most correlated to taxonomic distribution across samples. These results expand our understanding of microbial resource utilization within hypersaline lakes and the potential adaptations of dominant microorganisms that allow them to inhabit such environments.

Key words: hypersaline environment; Bacteria; Archaea; community structure; microbial diversity

Introduction

Hypersaline environments such as hypersaline lakes, solar salterns and ponds, springs, soils, and rock salt deposits are widely distributed around the world (Oren et al. 2011). There are three general salt-type environmental domains based on the differing saturation of salts: carbonate (70–140 g/L), gypsum (220–290 g/L) and halite (>290 g/L) domains (Ventosa et al. 2014). Studies of hypersaline ecosystems have mainly focused on solar salterns and saline lakes including the Dead Sea, the Great Salt Lake, Middle Eastern soda saline lakes, and African and Antarctic saline lakes (Tazi et al. 2014; Ventosa et al. 2014; Abdallah et al. 2016). A diversity of halophiles inhabit hypersaline lakes, and consist of all taxonomic domains including Bacteria, Archaea, viruses and eukaryotes (Emerson et al. 2013; Oren 2014). However, the distribution of taxa can vary considerably due to the differing characteristics of saline environments. As salinity increases, Archaea dominate over Bacteria, especially at salinities above 25% (Simachew et al. 2016). Moreover, many of the Bacteria and Archaea that are detected in such environments are phylogenetically novel organisms or have previously not been known to exist in these environments (Ghai et al. 2011). In general, microbial community composition across samples is commonly found to correlate to salinity levels (Çınar and Mutlu 2016; Simachew et al. 2016).

Typical bacterial phyla that are found in these environments include Proteobacteria (α -, γ - and δ -proteobacteria), Bacteroidetes, Firmicutes, Actinobacteria, Deinococcus-Thermus and Verrucomicrobia (Simachew et al. 2016; Horikoshi et al. 2010). Typical Archaea found in hypersaline environments belong to the *Halobacteria* class of the *Euryarchaeota* phylum (especially to the order *Halobacteriales*, *Haloferacales* and *Natrialbales*), in addition to other Archaea in the families of *Methanosarcinaceae* and *Methanocaldococcaceae* (Abdallah et al. 2016; Kambura et al. 2016; Thombre et al. 2016;). Currently, the *Halobacteriales* order contains three families, which are *Halobacteriaceae* (including *Halobacterium*, *Natronoarchaeum*, *Halovenus* and *Salinarubrum*, etc., distributed in forty-eight validly described genera), *Haloarculaceae* and *Halococcaceae*. The

Haloferacales order contains two families (Halorubraceae and Haloferacaceae) and the Natrilbales order contains only one family (Natrilbaceae) (Amoozegar et al. 2017; Sorokin et al. 2017).

The Qaidamu Basin of the Qinghai-Tibetan Plateau is known for its abundance of salt lakes, which cover the largest area of any region in the world ($2 \times 10^6 \text{ km}^2$), occur at the highest altitude (average $>4500 \text{ m}$) and consists of thousands of lakes (Liu et al. 2014; Zhong et al. 2016). Qaidamu lakes are characterized by low nutrient content, low temperature, low productivity, and high exposure to UV radiation (Guan et al. 2013). The microbial community structure, resource diversity and intracellular secondary metabolite availability of brackish and freshwater lakes of the Qinghai-Tibet Plateau, including Qinghai Lake, Namtso Lake, Pumoyum Co Lake and Awengcuo Lake, have been studied previously (Jiang et al. 2006; Jiang et al. 2009; Hu et al. 2010; Jiang et al. 2010; Xiong et al. 2012; Liu et al. 2014; Zhong et al. 2016). However, little is known about the patterns of bacterial and archaeal community diversity across gradients in salinity and other physiochemical parameters of inland plateau lakes, and particularly of magnesium sulfate-subtype hypersaline lakes.

The physicochemical characteristics of hypersaline lakes are highly variable, which affects the distribution and diversity of halophilic bacteria and archaea (Mesbah et al. 2008; Tazi et al. 2014). Keke Salt Lake ('KSL') is located in the Qaidamu Basin, features a continental arid climate, and has unique brine hydrochemical characteristics due to high concentrations of magnesium in the form of MgCl_2 and MgSO_4 (Zheng and Liu 2009). These characteristics differentiate KSL from other hypersaline lakes and brackish lakes of the region. Novel, moderately halophilic bacteria, *Gracilibacillus kekensis* sp. and *Salinicoccus kekensis* sp., have been previously isolated from this lake (Gao et al. 2010; Gao et al. 2012). However, the microbial structure and diversity has not been investigated here, nor did in these types of hypersaline lakes. High-throughput sequencing has recently been adopted to survey microbial communities via 16S rRNA gene composition in natural environments. These methodologies have become increasingly important for determining differences in microbial community diversity and structure, and in understanding microbial interactions and

adaptations to environments (Wolfe et al. 2014; Zhang et al. 2016). The present study analyzed the microbial structure of halophilic Bacteria and Archaea in KSL using high-throughput 16S rRNA gene sequencing of communities in order to better understand microbial resource use in these environments and adaptations towards extreme hypersaline environments.

Materials and methods

Sample sites and collection

Keke Salt Lake ('KSL'; 98° 15' 3.4", 36° 58' 19.97") locates in the northeastern margin of the Tibetan Plateau at an altitude of 3010 m, comprises an area of 116 km² and featuring an arid or semiarid continental climate. KSL is surrounded by the Qilian, Kunlun, and Aejin Mountains. The brine water of KSL has a relative density of 1.22-1.24, a total salinity of 326.4g/L, a pH of 6.75, a maximum depth of 0.5 m, and is characterized as a magnesium sulfate-subtype hypersaline lake based on its brine hydrochemical characteristics (Zheng and Liu 2009). The salt lake basin is a closed ecosystem, without the influx of perennial rivers. Consequently, the replenishment of water relies on the atmospheric precipitation and groundwater recharge, especially on the distribution of 665 underground springs. KSL displays a solid-liquid halite coexisting characteristic, possessing a total salt reserve of 1.03 billion ton. Halite mine is layered, and divided into six floors with an average thickness of 9.48 m. The representative mineral assemblages include halite, mirabilite, bloedite, gypsum and epsomite. The brine water is further divided into two types: the surface brine and the intercrystalline brine, comprising of NaCl, Na₂SO₄, MgSO₄, CaSO₄, MgCl₂, KCl, CaCl₂ and insoluble substances.

Four samples were collected that consisted of mixtures of water, lake sediments and salt crystals (~ 10 L total) from 10–40 cm depth in mid-July of 2015 (**Fig.1**). The distance between each two sample points was greater than 1 km. Samples K1, K2 and K3 were collected from the salt crystal saturated zone at depths of 40 cm, 25 cm and 10 cm, and exhibited temperatures of 24.4 °C, 26.2 °C and 26.7 °C, respectively. Sample K4 was collected from the edge of KSL, approximately 5 m from

the shore, did not contain salt crystals, had a temperature of 27.1 °C, and was influenced by fresh water input. All samples were stored at 4 °C in the field, and were then transported to the lab immediately.

Hydrochemical analyses

Water samples for chemical studies were filtered with cellulose acetate membranes (pore size, 0.45µm). Ion chromatography was used to measure total salinity, major anion and cation concentrations (Na^+ , K^+ , Ca^{2+} , Mg^{2+} , Cl^- , CO_3^{2-} , SO_4^{2-} and NO_3^-), pH, total organic carbon (TOC) and total nitrogen (TN) at Shanghai Microspectrum Chemical Technology Service Co., Ltd (China). Chloride type, magnesium sulfate subtype, sodium sulfate subtype or carbonate type are divided according to the Kurnakov-Valyashko classification and the Zheng's sub-classification (Zheng and Liu 2009), based on the analysis of the chemical classification of salt lake.

Genomic DNA extraction and quality assessment

Microbial community samples were obtained by vacuum filtration of mixtures using a 0.22 µm filter membrane. Filter membranes were then sectioned and community DNA was extracted using the Genomic QIAamp Fast DNA Stool Mini Kit (Qiagen, Hildesheim, Germany) according to the manufacturer's instructions. Extracted DNA integrity was then assessed using 1.0% agarose gel electrophoresis and DNA purity was further assessed using a Nanodrop 2000 (Thermo Fisher, USA). Extracted DNA was stored at -80°C.

16S rRNA gene PCR amplification

Archaeal and bacterial community composition was assessed using domain-specific primers in PCR amplification. The V3-V4 region of bacterial 16S rRNA genes were amplified using the barcoded primers 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGAC TACHVGGGTWTCTAAT-3') (Yu et al. 2005), while PCR amplifications of the V3-V5 region of archaeal 16S rRNA genes employed the barcoded primers Arch-344F (5'-ACGGGGYGCAGCAGGCGCGA-3') and Arch-915R (5'-GTGCTCCCCCGCCAATTCCT-3')

(Ohene-Adjei et al. 2007). PCR mixtures (final volume, 100 µl) contained 20 µl of 5x reaction buffer (Trans Start™ Fast Pfu Buffer, TransGen Biotech, Inc. China), ~50 ng of DNA, 20 mmol of each primer, 2.0 U Pfu polymerase (Trans Start™ Fast Pfu DNA Polymerase, TransGen Biotech, Inc. China), and 0.5 mmol dNTPs. For each sample, three replicate PCRs were performed using an ABI GeneAmp® 9700 PCR System (Applied Biosystems, CA, US). PCR conditions were as follows: 95 °C for 3 min; 27 (Bacteria) and 32 (Archaea) cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s, which was followed by a final elongation step at 72 °C for 10 min. PCR amplicons were purified using an AxyPrep™ DNA Gel Extraction Kit (Axygen, USA) after visualization on a 2% agarose gel using electrophoresis (supplemental material Fig. S1).

Sequencing optimization

Quantification of PCR products was conducted using a Quanti Fluor™-ST Fluorometer System (Promega Corporation, USA), followed by pooling in equimolar ratios into a single amplicon pool according to the manufacturer's instructions. High-throughput sequencing of 16S rRNA gene amplicons was performed on an Illumina MiSeq 300PE platform at Shanghai Majorbio Bio-pharm Technology Co., Ltd. (China). The number of paired-end reads for each sample ranged from 30,000 to 50,000. Fast length adjustment of short reads (FLASH) (Magoč and Salzberg 2011), was used to merge the raw paired-end reads into contig sequences based on sequence overlap (the shortest overlap length was 10 bp). The Trimmomatic software version 0.36 was used to parse sequences into their respective samples based on their unique barcodes (Bolger et al. 2014). Low quality sequences were discarded based on the following criteria: (i) 250 bp reads were truncated at any site with an average quality score <20 over a 10 bp sliding window, and the truncated reads were shorter than 50 bp, (ii) >0 mismatches to barcodes, >1 mismatch to the primer, and reads with ambiguous bases, (iii) ≤10 bp overlap in the forward and reverse reads. Unassembled reads were discarded.

Community diversity and structure analyses

UCLUST was used to cluster sequences into Operational Taxonomic Units (OTUs) at $\geq 97\%$ 16S rRNA gene sequence similarities (Ramette 2009). Chimeric OTUs were removed from the dataset using Usearch 7.1 (Edgar 2013) and by comparing sequences against the SILVA database release 119 (Quast et al. 2013), Greengenes database 13.5 (DeSantis et al. 2006) and RDP database version 11.3 (Cole et al. 2013). Taxonomic assignments of OTUs were performed using the Quantitative Insights into Microbial Ecology (QIIME) pipeline and the RDP classification method (version 2.2) using a 70% confidence level cutoff for assignment (Caporaso et al. 2010).

Statistical analyses

A number of alpha diversity measures were assessed using the Mothur software package version 1.30.1 (Schloss et al. 2009) including rarefaction analyses, the abundance based coverage estimator (ACE), terminal richness estimation (Chao1), the Shannon-Wiener index, the Simpson index and the Good's coverage estimation. A heat map was constructed to visualize relative abundance differences (P value cutoff of 0.001) using the Vegan Package for R. Beta diversity was measured using Bray-Curtis distances among samples and community differences were assessed using complete-linkage clustering analysis. Canonical correspondence analysis (CCA) was performed using the CCA function in the vegan R-package using the community distance matrices and water environmental factors. Variables that significantly explained community compositional variation were assessed using permutation tests under a reduced model (Hejmanová-Nežerková and Hejman 2006).

Sequence accession numbers

Raw 16S rRNA gene sequences were deposited in the NCBI database under the BioSample accession numbers SAMN04932686 to SAMN04932690 for Bacteria, and SAMN05356254 to SAMN05356257 for Archaea.

Results

Hydrochemical characteristics

Physicochemical parameters were distinct among the four hypersaline KSL samples (**Table 1**). In the K1, K2, and K3 samples, salinity ranged from 299.97 to 327.26 g/L and exhibited halite domain characteristics, while Mg^{2+} concentrations ranged from 25.21 to 27.39 g/L which are typical of magnesium sulfate-subtype environments (identified with the coefficient $Kn_1 = ([CO_3^{2-}] + [HCO_3^-]) / ([Ca^{2+}] + [Mg^{2+}]) \leq 1$, the $Kn_2 = ([CO_3^{2-}] + [HCO_3^-] + [SO_4^{2-}]) / ([Ca^{2+}] + [Mg^{2+}]) \leq 1$ and the $Kn_3 = [SO_4^{2-}] / [Ca^{2+}] \geq 1$). The pH ranged from 6.75 to 6.86, and was slightly acidic, which was likely due to the dissociation equilibrium of high Mg^{2+} concentrations ($[Mg^{2+}] + H_2O \rightleftharpoons Mg(OH)_2 + [H^+]$) and/or spring water inputs. In sample K4, ionic composition (e.g. Na^+ , K^+ , Ca^{2+} , Mg^{2+} , Cl^- , CO_3^{2-} and SO_4^{2-}) was significantly lower than in the other samples whereas total organic carbon (TOC) and total nitrogen (TN) was higher and likely due to input of freshwater and associated nutrients.

16S rRNA gene sequencing

Community composition was investigated in the four KSL samples by high-throughput Illumina sequencing. A total of 1481 OTUs were recovered comprising 734 and 747 bacterial and archaeal OTUs, respectively. Rarefaction analyses were used to compare richness among samples, and assess whether community diversity was adequately sampled. Rarefaction curves indicated similar overall diversity saturation patterns for all samples (**Fig. S2**), while OTU diversity was significantly lower in the K1, K2, and K3 samples that were collected from the salt-saturated area of the salt lake compared to the near-shore K4 sample.

Microbial diversity analysis

Diversity, as measured by OTU counts, the Ace index, Chao1 richness estimation, Good's Coverage, the Shannon-Weaver index and the Simpson index was provided in **Table 2**. Richness and diversity in the near-shore, low-salinity (~10%) sample K4 was remarkably higher than the salt-saturated samples K1, K2, and K3, which exhibited 30% or higher salinity. The diversity and composition of microbial communities varied with sample depth, indicating differential vertical

distribution of microbial taxonomic diversity. Bacterial OTU richness (94, 115 and 132 OTUs) decreased with depth in samples K1, K2, and K3, respectively, while archaeal richness increased with depth: 121, 104, and 77 archaeal OTUs in K1, K2 and K3, respectively. Although overall microbial diversity in extremely hypersaline environments were distinctly less than that of more mesic environments, archaeal diversity was high, as noted by several diversity metrics, indicating that Archaea are particularly diverse in this environment, relative to Bacteria.

Taxonomic composition of KSL microbial communities

Twenty-one bacterial phyla were detected in the KSL microbial communities and consisted of 43 classes and 201 genera. Four archaeal phyla were detected that comprised four classes and 39 genera. Relative abundances of bacterial and archaeal populations at the phylum, class and genus level were shown in **Fig. 2**. The dominant bacterial phyla (>1% relative abundance in any sample) in the hypersaline K1, K2, and K3 samples belonged to the Firmicutes (77.21%–80.03%), followed by the Proteobacteria (15.50%–19.27%), Bacteroidetes (2.68%–3.27%) and Actinobacteria (0.70%–2.45%). Together, these bacterial phyla constituted more than 99.9% of all the reads assigned at the phylum level (**Fig. 2A**). Sample K4 was considerably different than the other samples and more diverse, with dominant populations belonging to the phyla Proteobacteria (34.42%), Firmicutes (29.36%), Bacteroidetes (17.60%), Actinobacteria (2.10%), Cyanobacteria (6.25%), Chloroflexi (1.78%) and Spirochaetae (1.61%), along with ~ 6.86 % of the reads that could not be classified at the phylum level.

Euryarchaeota was the dominant archaeal phylum in the K1, K2, and K3 samples and accounted for 87.26–98.48% of all reads in each sample, followed by unclassified Archaea (0.96%–8.16%) and Nanohaloarchaeota (0.38%–4.58%). In the K4 sample, the uncultured Woesearchaeota DHVEG-6 group comprised 56.13% of the total archaeal community, followed by Euryarchaeota (36.87%), unclassified Archaea (5.08%), Nanohaloarchaeota (0.02%) and reads that could not be assigned to a known phylum (1.90%). Community compositional differences among the samples indicated that the

251 saline hydrochemical characteristics greatly affected community ecology.

252 At the class level (**Fig. 2C**), the dominant bacterial classes in the hypersaline K1, K2, and K3
253 samples belonged to the Bacilli (74.81–80.99%), followed by the Gammaproteobacteria
254 (15.29%–18.86%), Flavobacteria (2.67%–3.25%), Actinobacteria (0.70%–2.43%) and
255 Sphingobacteria (<0.05%). In addition to the above classes, Deltaproteobacteria (10.25%),
256 Alphaproteobacteria (6.40%), Cyanobacteria (4.11%), Cytophagia (3.54%), Spirochaetes (1.61%),
257 Anaerolineae (1.32%), unclassified Bacteroidetes (1.37%) and multiple candidate divisions (WS3
258 [1.19%], SB-1 [1.31%], and BD2-2 [1.87%]) were also detected in the K4 sample. The dominant
259 archaeal classes (**Fig. 2D**) of the K1, K2, and K3 samples were the Halobacteria (84.19%–93.02%),
260 followed by Methanobacteria (13.51%–24.33%), unclassified Nanohaloarchaeota (0.38%–4.58%),
261 Methanomicrobia (0.02%–4.90%), unclassified Archaea (0.96%–8.16%) and Thermoplasmata
262 (0.61%). The above-mentioned classes were present in sample K4a although the abundances were
263 significantly different compared to the hypersaline samples. Further, an abundant and unclassified
264 class of the Woese archaeota DHVEG-6 (56.13%) group was present in the K4a sample.

265 At the genus level (**Fig. 2E**), the dominant bacterial genera of the K1, K2, and K3 samples were
266 *Bacillus* (51.52%–58.35%), *Lactococcus* (9.52%–10.51%), *Pseudomonas* (9.15%–10.54%),
267 *Oceanobacillus* (8.82%–9.88%), *Stenotrophomonas* (3.17%–3.72%), *Psychrobacter* (2.07%–2.75%),
268 *Arthrobacter* (0.69%–2.36%), *Myroides* (1.85%–2.28%) and *Brochothrix* (1.56%–1.88%). In
269 addition to the aforementioned genera, other genera in the K4 sample included *Desulfotignum*
270 (2.32%), *Litoricola* (2.08%), *Microcoleus* (2.07%), *Marinicella* (1.29%), *Psychromonas* (1.24%),
271 *Spirochaeta* (1.23%), and *Marinobacter* (1.12%). Minor populations of unclassified or uncultured
272 genera were also present in K4 (*Rhodobacteraceae* [2.07%], *Chloroplast* [1.95%], *Saprospiraceae*
273 [1.67%], *Bacteroidetes* [1.37%] and *Anaerolineaceae* [1.27%]), as well as a considerable abundance
274 of candidate division Bacteria (ML602J-37 [2.01%], BD2-2 [1.87%], Sh765B-TzT-29 [1.60%], SB-1
275 [1.31%] and WS-3 [1.19%]).

In the salt-saturated K1a, K2a, and K3a samples (**Fig. 2F**), the dominant archaeal genera (>10% relative abundance in any sample) were the *Halonotius*, *Halorubellus*, *Halapricum*, *Halorubrum* and *Natronomonas*, each with widely varying abundance. Subdominant genera (5–10% relative abundance) consisted of *Halolamina*, *Halovenus*, *Haloplanus*, unclassified Archaea, and uncultured or unclassified genera in Halobacteriaceae. Lastly, a second subdominant group of genera (1–5% relative abundance) included *Haloquadratum*, *Haloarcula*, *Halobacterium*, *Halobellus*, *Halomarina*, *Methanobrevibacter*, *Halorhabdus*, *Candidatus Halobonum* and unclassified genera within the Nanohaloarchaeota. Other minor genera included *Haloterrigena*, *Halorientalis*, *Halomicroarcula* and *Halosimplex*, along with unclassified or uncultured genera that constituted small percentages of community abundances (<1.5%). In sample K4, the unclassified Woesearchaeota DHVEG-6 group (56.1%) was the dominant genus in the archaeal community, followed by *Haloarcula* (5.8%), *Halorubrum* (5.4%), unclassified Archaea (5.1%), unclassified genus within the Halobacteriaceae family (4.3%), *Haloterrigena* (2.5%), *Haloterrigena* (2.4%), *Halorubellus* (2.0%), *Halolamina* (2.0%), *Halapricum* (1.9%) and *Natronomonas* (1.8%).

Cluster analysis of OTU distributions

Cluster analysis was performed based on OTU relative abundances in order to discern community compositional differences among samples. Clustering of bacterial communities showed that the Firmicutes (e.g. *Bacillus*, *Oceanobacillus*, *Lactococcus*, *Brochothrix*, *Lysinibacillus* and *Streptococcus* within the Bacilli class), followed by Proteobacteria (e.g. *Pseudomonas*, *Stenotrophomonas*, *Psychrobacter*, *Acinetobacter*, *Rahnella* and *Enhydrobacter* within the γ -Proteobacteria), Bacteroidetes (*Myroides* and *Flavobacterium* within the Flavobacteria class) and Actinobacteria (*Arthrobacter* in the order Micrococcales) most differentiated samples (**Fig 3A**). In contrast, Euryarchaeota (*Halonotius*, *Halapricum*, *Haloplanus*, *Halorubrum*, *Halorubellus* and *Natronomonas* within the Halobacteria class) predominantly segregated archaeal communities. The overall taxonomic diversity of Archaea was considerably lower than that of the bacterial

communities (**Fig 3B**). Bacterial and archaeal taxonomic diversity was significantly lower in the salt-saturated K1, K2 and K3 samples compared to the near-shore K4 sample.

Relationships between environmental factors and community structure

Relevant environmental factors varied considerably with prokaryotic community composition dependent on the genera that were considered (**Fig 4**). The abundances of bacterial genera *Bacillus*, *Oceanobacillus* and *Lactococcus* of the Firmicutes phylum, and *Pseudomonas* of the Proteobacteria phylum, that were all dominant in the three hypersaline samples, were correlated with ionic concentrations (total salinity, $\text{Mg}^{2+}/\text{Cl}^-$ and Na^+/K^+ concentrations) and pH value. In contrast, four of the bacterial clusters in the K4 sample (uncultured Saprospiraceae, an unclassified genus of Bacteroidetes, *Microcoleus* and *Spirochaeta*, among others; dashed circle in **Fig 4A**) were significantly associated with TN and TOC. Total salinity was the most important impact factor for determining species composition of all variables tested. As expected, various ionic concentrations were autocorrelated with total salinity, including Mg^{2+} , Cl^- , Na^+ and K^+ . Archaeal community structures were most strongly correlated to total salinity and other secondary factors (Mg^{2+} , Cl^- , Na^+ , K^+ , pH, Ca^{2+} and SO_4^{2-}). For example, the genera *Halovenus*, *Salinarchaeum*, *Halapricum*, *Halobellus*, *Halolamina*, *Halorubrum*, *Methanobrevibacter*, *Halorhabdus* and *Haloquadratum*, which were all primarily found in the salt-saturated K1, K2, and K3 samples were particularly associated with the above environmental variables. TOC, TN and CO_3^{2-} exhibited significant positive correlations with the relative abundances of *Haloarcula* and the Woese archaeota DHVEG-6 group, which were particularly prevalent in the K4a sample (**Fig 4B**).

Discussion

Hypersaline characteristics of KSL

Brackish (low salinity: 1.0–35.0 g/L), mesosaline (medium salinity: 35.0–50.0 g/L) and hypersaline lakes (total salinity >50.0 g/L) are widely distributed in northwestern China, and exhibit varied brine hydrochemical characteristics (Zheng and Liu 2009). KSL exhibited a halite type

salinity of 299.97–327.26 g/L, slightly acidic pH of 6.75–6.86, and Mg^{2+} concentrations of 25.21–27.39 g/L (**Table 1**). These hydrochemical characteristics were somewhat similar to the Dead Sea, another example of a MgSO_4 -subtype hypersaline lake, which featured a salinity of ~347 g/L, a slightly acidic pH (6.0) and a Mg^{2+} concentration of 48.0 g/L, in addition to Chott El Jerid (salinity: 346 g/L, pH: 6.61–6.90, Mg^{2+} : 12.0 g/L) (Bodaker et al. 2010; Abdallah et al. 2016). However, ionic composition, major ion content and pH of KSL were considerably different from other types of hypersaline environments, including the Santa Pola salterns in Spain (Ghai et al. 2011), Tyrrell Lake in Australia (Narasingarao, et al. 2012), Great Salt Lake (Tazi et al. 2014) and other mesosaline lakes in the Qinghai-Tibet Plateau where previous studies have mostly focused on freshwater or brackish lakes.

Microbial community structure of KSL

The microbial community structure in the hypersaline KSL was comprehensively investigated for the first time using high-throughput sequencing of community 16S rRNA genes. The bacterial community compositions and especially the dominant phylum of KSL were clearly different than other hypersaline lakes in the Qinghai-Tibet Plateau, which may be due to the differing hydrochemical characteristics. The bacterial phyla of Xiaochaidan Salt Lake (salinity: 95.0–99.0g/L, pH: 7.9–8.4) consisted of Proteobacteria (33.0%), Actinobacteria (31.0%), Bacteroidetes (17.0%), Verrucomicrobia (8.0%) and Planctomycetes, along with minor abundances of the phyla Cyanobacteria and Tenericutes (Zhong et al. 2016). In Gasikule Lake (salinity: 317–344 g/L, pH: 7.2–7.3), seven phyla were detected, and primarily comprised the archaeal phylum Euryarchaeota (83.0%), with the remainder being comprised of bacterial phyla: Bacteroidetes (14.0%), Proteobacteria (2.0%), and 0.5% consisting of Actinobacteria, Verrucomicrobia, Planctomycetes and Cyanobacteria (Wang et al. 2014). Similarly, eight bacterial phyla were found in Zhacang Chaka Lake (salinity: 308.0 g/L, pH: 9.5), and included Proteobacteria (23.74%), Firmicutes (20.63%), Bacteroidetes (12.97%), Deinococcus-Thermus (4.41%), Actinobacteria, Chloroflexi, Cyanobacteria

and Acidobacteria (Xiong et al. 2012).

Here, in the salt-saturated samples of KSL (**Fig. 2AB**), the primary bacterial phyla were Firmicutes (77.21%–80.03% relative abundance), Proteobacteria (15.50%–19.27%), Bacteroidetes and Actinobacteria, with the Euryarchaeota primarily comprising most of the archaeal communities (87.26–98.48%). These results demonstrated that the bacterial community structure and the dominance of Firmicutes in KSL were inconsistent with results from other hypersaline lakes. In contrast, the dominance of Euryarchaeota among archaeal communities was consistent with numerous studies of hypersaline lakes (Jiang et al. 2009).

Dominance of Firmicutes in KSL

Organisms within the phylum Firmicutes were typical and dominant bacteria among MgSO₄-subtypes hypersaline KSL samples. The two predominant classes in hypersaline Firmicutes are the Bacilli (including the families Bacillaceae, Planococcaceae and Staphylococcaceae) and the Clostridia (including the families Halanaerobiaceae and Halobacteroidaceae) (Horikoshi et al. 2010). Here, we found that the most dominant genus within the Firmicutes component of the KSL hypersaline communities was the genus *Bacillus* (74.81–80.99%), followed by *Lactococcus* (9.52%–10.51%) and *Oceanobacillus* (8.82%–9.88%), while several other genera (including *Brochothrix*, *Lysinibacillus* and *Streptococcus*) accounted for minor community proportions (**Fig. 2E**). In other hypersaline lakes such as Chott El Jerid Lake, the Dead Sea and the Great Salt Lake, the genus *Salinibacter* predominates (Bodaker et al. 2010; Abdallah et al. 2016; Tazi et al. 2014), but was not prevalent in KSL. Thus, the genera *Bacillus*, *Lactococcus* and *Oceanobacillus* might be considered unique representative bacteria of KSL (**Fig. 3A**).

Bacterial genera unique to the KSL

The dominant bacterial genus in hypersaline KSL samples was *Bacillus*, which mostly comprised six species, where most belonged to unclassified *Bacillus* (51.24%–58.05% relative abundance), followed by *B. oceanisediminis* and *B. badius*, which both were generally <0.5% relative abundance

of communities. Many members of the genus *Bacillus* are capable of withstanding extreme conditions, owing to facultative anaerobic physiologies, and especially their capability of producing endospores (Horikoshi et al. 2010).

The second-most abundant genus that was unique to KSL was *Lactococcus*, which has been scarcely detected in hypersaline lakes. The genus was most-represented in KSL by *L. piscium* (6.19%–7.06% relative abundance), followed by other unclassified *Lactococcus* spp. (3.25%–3.45%). *L. piscium* is a psychrotrophic lactic acid bacterium, capable of surviving and proliferating at low temperatures (even at 0 °C) (Saraoui et al. 2016). Cold adaptations could be advantageous for bacterial competition in an extreme cold environment such as KSL which experiences average temperatures of –14°C in January.

A third bacterial genus that was dominant in KSL but not present in other hypersaline lakes was *Oceanobacillus*, which was represented by *O. profundus* (8.79%–9.84%) and a minor fraction of unclassified *Oceanobacillus* spp. The *Oceanobacillus* genus consists of Gram-positive, endospore-forming, and moderately halophilic bacteria, which are frequently isolated from hypersaline lakes, salty lakes, marine sediments and seawater (Amoozegar et al. 2016).

Dominant Halobacteriaceae in KSL

The dominance of Halobacteriaceae within the phylum Euryarchaeota in KSL samples is consistent with their dominance in hypersaline environments (Çınar and Mutlu 2016). Twenty-two genera that are affiliated with the Halobacteriaceae were detected in KSL, including the genera *Halonotius*, *Halorubellus*, *Halapricum*, *Halorubrum* and *Natronomonas*, which each comprised >10% of the archaeal communities (**Fig. 2F**). Baricz *et al.* (2014) analyzed the archaeal diversity of the hypersaline lakes Ocnei (salinity: 322 g/L, pH: 7.2–8.4), Brâncoveanu (salinity: 283–331 g/L, pH: 7.2–8.6) and FărăFund (salinity: 315–330 g/L, pH: 6.0–8.5) in Romania, and found that the most dominant genera were *Haloferax* (47%), *Halobacterium* (12%) and *Halorubrum* (11%). Our results are consistent with the general dominance of Halobacteriales of hypersaline lakes, but importantly,

the dominant genera were distinct from other lakes (Jiang et al. 2006; Çınar and Mutlu 2016).

The class Halobacteria only consists of the Halobacteriaceae family, which comprises 52 genera and 215 species (Oren and Ventosa 2016). All of the genera and species in Halobacteriaceae exhibit strict anaerobic and heterotrophic physiologies (Seckbach et al. 2013). Notably, nearly 5% of the KSL archaeal communities were affiliated with the recently described '*Candidatus Haloredividus*' (phylum Nanohaloarchaeota), which consists of ultrasmall, uncultivated archaeal lineages that were previously detected in other salt-saturated environments (Meglio et al. 2016), but have not been previously detected in the Qinghai-Tibet Plateau.

Woesearchaeota in the KSL

Of particular interest, the dominant Archaea in sample K4a belonged to the Woesearchaeota DHVEG-6 group (a total of 329 OTUs and ~56.1% relative abundance), which has not been previously reported from samples in the Qinghai-Tibet Plateau (Jiang et al. 2009; Jiang et al. 2010). DHVEG-6 Archaea have also been previously detected in the Xiaochaidan Salt Lake (salinity: 95.0–99.0 g/L, pH: 7.9–9.2) at considerable abundances 75.64%–85.83% (our studies data but not published). The Woesearchaeota phylum within the proposed DPANN superphylum (Diapherotrites, Parvarchaeota, Aenigmarchaeota, Nanohaloarchaeota and Nanoarchaeota) of the Archaea have been primarily reported from saline habitats and sediments, including oceans, hydrothermal vents, and continental and high-altitude lakes (Eme and Doolittle 2015; Ortiz-Alvarez and Casamayor 2016). The growth of Woesearchaeota has also been positively correlated with oligotrophic plateau lakes that overall very poor in nutrients (Ortiz-Alvarez and Casamayor 2016). Metabolic reconstructions of Woesearchaeota genomes have indicated that most of their core biosynthetic pathways are only partially present or entirely absent, suggesting possibly symbiotic or parasitic lifestyles (Castelle et al. 2015). Therefore, the presence of Woesearchaeota in the K4a sample likely reflects the oligotrophic nature of KSL resulting from the geographic and hydrochemical characteristics of the Qinghai-Tibet Plateau.

Environmental factor and dominant microbes

Total salinity (consisting of Mg^{2+} , Cl^- , Na^+ and K^+) is one of the most important environmental factor correlated to distributions of dominant bacterial and archaeal OTUs (Xiong et al. 2012; Liu et al. 2014). Here, the bacterial genera that are characteristic of KSL (*Bacillus*, *Lactococcus*, *Oceanobacillus* and *Pseudomonas*) and the dominant archaeal genera (*Halovenus*, *Salinarchaeum*, *Halapricum*, *Halobellus*, *Halolamina*, *Halorubrum*, *Methanobrevibacter*, *Halorhabdus* and *Haloquadratum*) were highly correlated to concentrations of Mg^{2+} and Cl^- , followed by Na^+ and K^+ concentrations in the salt-saturated KSL samples (Fig 4). These results are in agreement with the recent work of Zhong et al (2016) on Tibetan Plateau Lakes, although the distribution of dominant taxa differed in the hypersaline KSL. The MgSO_4 -subtype KSL had considerably higher Mg^{2+} concentrations in the brine compared to other hypersaline lakes, which is toxic towards microbial growth at high concentrations. However, our results may indicate possible adaptations of the dominant Archaea and Bacteria in order to survive in such a harsh and hypersaline environment.

Uncultured microorganisms in KSL

A large number of unclassified Bacteria and Archaea were also detected in KSL, with the abundance of unclassified microorganisms at the phylum being 3.4% and 56.3%, respectively. Unclassified Bacteria consisted of the novel candidate divisions BRC1, BD1-5, JS1, OD1, WS6, SBYG-2791 and NPL-UPA2, whereas the archaeal candidate divisions SM2F11, SM1K20 and DHVEG-6 were prevalent. The particularities of their ecological and biological interactions are not known in the hypersaline high-altitude lakes, probably because the genetic mechanisms do not have homologues or orthologues in the currently known organisms. Therefore, experiments with pure isolates from lake samples under controlled laboratory conditions are under way and will help to clarify the presence of critical resources for novel taxonomic diversity.

Conclusions

KSL is a typical MgSO_4 -subtype hypersaline lake in the Qinghai-Tibet Plateau and serves as an

excellent model for understanding the microbial diversity and adaptations to hypersaline habitats. The bacterial and archaeal diversity in KSL was specific to this environment and contrasts with previous reports of similar habitats. High-throughput sequencing of 16S rRNA genes indicated that salt-saturated samples of KSL were dominated by the Firmicutes (primarily in the *Bacillus*, *Lactococcus* and *Oceanobacillus* genera) and the Euryarchaeota (mainly comprising the archaeal genera *Halonotius*, *Halorubellus*, *Halapricum*, *Halorubrum* and *Natronomonas* within the Halobacteriaceae family).

Of note, the presence of a high abundance of Firmicutes may indicate that the MgSO₄-subtype characteristics of KSL distinguished this environment from other hypersaline lakes. Our high-throughput sequencing analysis also provided evidence for the Nanohaloarchaeota and Woesearchaeota in Qinghai-Tibet Plateau lakes, which has not been previously documented. Total salinity (especially concentrations of Mg²⁺, Cl⁻, Na⁺ and K⁺) was found to be the primary environmental factor that correlates to community composition and taxonomic distribution at the level of individual clades as well as entire communities. Further studies are needed to reveal the functional roles of the dominant Bacteria and Archaea that inhabit MgSO₄-subtype hypersaline lakes such as KSL.

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Table 1. Chemical characteristics of surface water and sediment samples from the KSL.

Sample	Total salinity(g/L)	Temperature (°C)	pH	TOC (%)	TN (%)	Major ions (g/L)						
						Na ⁺	K ⁺	Mg ²⁺	Ca ²⁺	Cl ⁻	SO ₄ ²⁻	CO ₃ ²⁻
K1/K1a	327.26	24.4	6.86	2.89	0.49	80.05	4.62	27.39	0.23	187.52	23.43	0.31
K2/K2a	302.45	26.2	6.78	2.47	0.49	79.56	4.57	27.35	0.17	186.34	22.78	0.28
K3/K3a	299.97	26.7	6.75	2.31	0.48	62.15	4.42	25.21	0.11	165.13	18.43	0.27
K4/K4a	98.12	27.1	6.58	12.35	5.53	20.15	1.23	10.82	0.08	54.39	14.35	0.43

Table 2. OTU richness and diversity measures

Sample number		Sample depth	Number of Reads	Richness and Diversity Metrics					
				OTUs	ACE	Chao 1	Coverage	Shannon	Simpson
Bacteria	K1	40 cm	35,712	94	112±20	111±15	99.91	2.26±0.02	0.173±0.003
	K2	25 cm	28,357	115	127±7	128±9	99.92	2.49±0.02	0.143±0.002
	K3	10 cm	36,278	132	148±9	149±11	99.89	2.51±0.02	0.140±0.003
	K4	10 cm	33,515	674	725±20	757±35	99.61	5.09±0.03	0.023±0.001
Archaea	K1a	40 cm	24,544	121	130±6	137±12	99.90	3.69±0.02	0.043±0.003
	K2a	25 cm	24,564	104	110±4	113±7	99.92	3.69±0.02	0.047±0.002
	K3a	10 cm	28,634	77	85±6	83±5	99.94	3.66±0.02	0.049±0.001
	K4a	10 cm	35,630	652	699±17	702±21	99.39	5.38±0.02	0.010±0.001

Fig.1 Map of Keke Salt Lake (KSL) showing sampling locations. (A) Geographic location of KSL and four samples K1, K2, K3 and K4. (B) Location of KSL in the Qaidamu Basin of Haixi Prefecture, Qinghai Province. (C) Location of KSL in the northwest of China.

144x113mm (96 x 96 DPI)

Fig.2 Bacterial and archaeal composition of Keke Salt Lake. A, C and E show bacterial taxonomic abundances of the four samples at the phylum, class, and genus level, respectively; B, D and F show archaeal taxonomic abundances of the four samples at the phylum, class, and genus level, respectively.

391x373mm (96 x 96 DPI)

Fig.3 OTU abundances and community cluster analysis of KSL samples. A shows the distribution of bacterial OTUs while B shows the distribution of archaeal OTUs, classified at the genus level. Colors correspond to the relative abundance of each OTU within the community, as given by the scale below the heatmap. Cluster distance is based on Bray-Curtis distances and the dendrogram was constructed using complete linkage clustering. Only the most abundant 100 genera based on statistical analysis are shown.

732x647mm (96 x 96 DPI)

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Fig. S1 Gel electrophoresis of 16S rRNA gene amplification products of Bacteria and Archaea in Keke Lake. Marker: DL2000; Lanes 1–4: amplification of the V3–V4 region of bacterial 16S rRNA genes of samples K1–K4, respectively; Lanes 5–8: amplification of the V3–V5 region of archaeal 16S rRNA genes of samples K1–K4, respectively; CK: negative control.

Fig. S2 Rarefaction curves depicting diversity of OTUs as a function of 16S rRNA gene sequencing effort. Samples K1–K4 corresponded to bacterial communities while samples K1a–K4a corresponded to archaeal communities.