Metagenomic Analysis Reveals the Shared and Distinct Features of Bacterial Communities and Resistomes in Corn Silage from Different Climate Zones in China

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Research Article

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Abstract

Background: The emergence and spread of antibiotic resistance are a significant threat to global health. Silage is a major forage feed for ruminants, and its safety is an important guarantee that high-quality ruminant products will remain available to humans. However, little attention has been given to the silage resistome. To define the antibiotic resistome and its potential risk to silage from different climate zones and in response to the ensiling process, this study used metagenomics to investigate bacterial communities and the type and amount of antibiotic resistance gene (ARG) in corn silage harvested from six climate zones (Cfa, BWk, Dwc, Dwa, BSk, and Aw based on Köppen-Geiger climate classification) in China.

Results: The composition and succession of silage bacterial communities varied significantly between different climate zones. *Lactobacillus* was the predominant bacteria during corn ensiling. A total of 134 ARGs were observed in corn silage, with the dominant classes being beta-lactamase and multidrug resistance and the primary mechanisms being efflux pump, inactivation, and target protection. Differences in the resistome were mainly attributed to disparities in microbial composition, which was indirectly affected by climatic factors and fermentation pH. ARG abundance was lower in 90-day silages than 5-day silages except in Hainan silage. The diversity and relative abundance (0.65-0.4% based on total gene number) of ARGs was lower in silage microbiota from Tibet than other climate zones. The dominant ARGs were *tetM*, *oqxB*, *lmrD*, *lnuA*, *ermB*, and *tetS*, and *Enterobacter, Klebsiella, Staphylococcus, Lactobacillus* and *Lactococcus* were the primary ARG hosts. Eleven high-risk ARGs were chosen to evaluate the pollution level of silages harvested from different climate zones. The highest relative abundance of high-risk ARGs belonging to *Lactobacillus* occurred in corn silages from Cfa, Dwa and BWk climate zones.

Conclusions: The ensiling process decreased ARG abundance. While resistome contamination of silage from Tibet was relatively low, ARGs with high risk were abundant in silages from Cfa, Dwa and BWk climate zones.

Introduction

Antimicrobial resistance (AMR) is a global health crisis and an estimated ten million deaths a year will be caused by 2050 if urgent action is not taken[1]. The increasing germination of AMR is attributed to the continuous use of antimicrobials in human and animal disease treatment, animal production, and agriculture[2]. Since the interplay between humans, livestock, food, and the environment is critical to AMR development[3], there is an urgent need to monitor its origin. The potential sources of AMR in animals and agriculture are a focal point for research[4]. Resistance is strongly associated with antimicrobial resistance genes (ARGs) and other acquired mechanisms. The spread of antimicrobial-resistant bacteria (ARB) and ARGs indicates that humans, animals, and the environment are connected and that a “One Health” approach is required for an effective public health response[5, 6].
The global population is predicted to surpass nine billion by the middle of the twenty-first century, and food demand is projected to grow by 70% by 2050[7]. In addition, it is estimated that most of the ruminant meat (50%) and milk (67%) in demand are produced in developing countries, in particular China and India[8]. Corn silage is a major source of energy, nutrients, and digestible fiber and a primary food ingredient for ruminants, especially dairy cows, which accounts for more than 40% of their daily ration[9]. Hence, nutritional and safe silage production is essential for ruminant husbandry to efficiently provide high-quality products to the growing global population. While studies have focused on improving the fermentation quality and feed efficiency of silage, few have evaluated the biosafety of silage such as AMR or ARGs. A previous study reported that ofloxacin and oxytetracycline could transfer from swine manure to the stem, leaves and root of alfalfa, while sulfamonomethoxine could only transfer to alfalfa roots[10]. While the ensiling process eliminated 68.7% of ofloxacin it only slightly decreased oxytetracycline levels. However, the impact of antibiotics on AMR or ARGs in silage remains unclear.

The fermentation process and quality of silage are largely shaped by the raw materials and epiphytic microbes. Climate is one of the primary factors affect the characteristics and phyllosphere microbial communities of forage before ensiling. China has many climate regions, ranging from tropical and subtropical in the South to cold in the North, and from humid in the East and South to arid and alpine in the West[11]. The bacterial community, fermentation characteristics, and biosafety of silage vary between different climate zones. Data on bacterial communities and ARGs of silage microbiota across these zones remain unclear. This study investigated the effect of different climate zones (Cfa, BWk, Dwc, Dwa, BSk and Aw based on Köppen-Geiger climate classification) on the bacterial communities and characteristics of ARG distribution during early and late fermentation stages of corn silage in China. Results described the microbiological process of corn silage fermentation and clarified the shared and distinct features of the corn silage resistome in different climate zones. These findings improve our understanding of silage microbiota and ARGs and allow for better evaluation of silage safety and the public health risk of ARGs in animal husbandry.

**Materials And Methods**

**Sampling sites and silage preparation**

Corn (*Zea mays* L. cv. Yuqingzhu No. 23) was planted in six provinces that represent different climate zones in China: Cfa (Guizhou), BWk (Xinjiang), Dwc (Tibet), Dwa (Heilongjiang), BSk (Shanxi), and Aw (Hainan), according to Köppen-Geiger climate classification[12] (Fig. S1). Whole-crop corn was harvested from three sites at early to middle dough stage (January 25, 2019 in Hainan and September 10 to October 10, 2018 in other regions) (Table S1) in a province with dry matter 215.7 to 279.4 g/kg fresh weight (215.7 in Tibet and 249.0 to 279.4 in other provinces). Whole-crop corn from each site was randomly collected from four cores in a planting field and chopped into 2-3 cm samples. Fresh chopped samples from each core were randomly divided into two sub-samples for fermentation times and four sampling cores were used for repetitions. The chopped whole-crop corn was ensiled in vacuum-sealed polyethylene plastic bags (30 cm × 45 cm) with about 500 g of fresh corn crops. A total of 144 bags were kept at
ambient temperature (22-25°C) and sampled after five and 90 days of fermentation. In 2018, meteorological information such as precipitation, mean temperature, mean relative humidity, and sunshine duration during the growing season of whole-crop corn from each sampling site was gathered from the China Meteorological Data Network (data.cma.cn) and the base stations of meteorological data collection were county level (Table S2).

**Fermentation quality analysis**

After five and 90 days of fermentation, the bags were opened and sampled for fermentation quality analysis. Twenty grams of fresh silage was homogenized with 180 ml distilled water in a juice extractor (BA-828, Mannengda Plasthetics Co. Ltd., Guangzhou, China) for 30 s at high speed. The pH of the filtrate was immediately detected with a glass electrode pH meter (PB-10, Sartorius, Germany) after filtration through four layers of sterile medical gauze. Fractions of the filtrate were acidified using 50% H$_2$SO$_4$ (w/w) at pH <2 and filtered through a 0.22 μm dialyzer. Lactic, acetic, propionic and butyric acid concentrations were detected using High-Performance Liquid Chromatography (HPLC, Agilent HPLC 1260 equipment, C-811 column, Shodex; Shimadzu: Japan; oven temperature 50°C; flow rate 1 ml/min; detection wavelength of SPD 210 nm) with acidified filtrate. A separate non-acidified filtrate was mixed with 250 g/l (w/vol) trichloroacetic acid at a ratio of 1:4 to precipitate true proteins (overnight at 4°C) and then centrifuged at 18,000 × g for 15 min at 4°C. The resulting supernatant was used to detect the concentration of ammonia nitrogen using the phenol-sodium hypochlorite method[13].

**DNA extraction, sequencing, and ARG analysis**

Corn silage after five and 90 days of fermentation from each climate zone was sampled for total DNA extraction. Prior to DNA extraction, corn silage samples from each silo bag were mixed thoroughly, and 20 g of fresh silage was homogenized in 50 ml of 1 × PBS and vigorously mixed for 5 min in a sterilized Erlenmeyer flask with a sterile sealing membrane. About 45 ml of suspension from every silage sample was collected and filtered through four layers of sterile medical gauze into sterile Falcon tubes. Filtrates were centrifuged at 5000 × g for 10 min at 4°C and the supernatant was discharged. After extraction of DNA from each silage sample, the extracted DNA from four cores were equally mixed into one sample for later analysis. Three sampling sites from each climate zone were considered repeats. A total of 36 DNA samples (0.2 μg) were collected (6 climate zones × 2 fermentation stages × 3 replications) for single-molecule real-time sequencing (SMRT) sequencing and metagenomic sequencing. The purity and completeness of DNA were evaluated using1.0% agarose gel. PCR amplification of the full-length 16S rRNA for SMRT sequencing was carried out with 27F and 1492R primers. The quality filter, cluster, sequence pre-processing, species annotation, and alpha diversity were performed as described by Xu et al.[14].

The metagenomic sequencing library generation was conducted using a DNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer's specification[15]. After library preparation, metagenomic sequencing was performed on an Illumina nova sequence platform using the 150 paired-end reads.
strategy. Quality control and host filtering of the raw data were performed and cleaned data (Clean Data) was analyzed using MEGAHIT software (--presets meta-large)[16]. The assembled Scaffolds were interrupted from the N junction to obtain sequence fragments called Scaftigs[17,18]. Filtered Scaftigs (>500 bp) were used for statistical analysis and gene prediction[19,20]. Gene prediction was performed as described by Wu et al.[15]. The Unigenes were compared with the Comprehensive Antibiotic Resistance Database (CARD) using BLASTN software to search for ARGs. Each compared sequence with an identity value greater than the minimum identity value required by the database was assessed to ensure the reliability of resistance gene annotation. The abundance of ARGs in each sample and the resistance mechanism of ARGs were conducted after filtration of comparison results. The ARGs and species annotation were conducted based on contigs. The chord diagrams were profiled using circos-0.69-9 software using the top ten ARGs (by relative abundance) to display of ARG hosts in corn silage across different climate zones.

Data analysis

The sensitive OTUs were identified as described by Hartman et al.[21]. In brief, the R package indicspecies was used to conduct indicator species analysis with $10^4$ permutations. P value <0.05 was considered as statistically significant. In addition, different OTUs were found in the diverse climate zones using likelihood ratio tests (LRT) method with the R package, edgeR, where the filtering threshold had a P-value <0.05. Sensitive OTUs were defined with confirmation of indicator species and LRT analysis.

Two co-occurrence networks of whole-crop corn fermented for five and 90 days were established. Spearman correlation between OTUs was conducted of networks, and positive and significant correlations were visualized ($p>0.7, P<0.01$). The networks were visualized using the R package, igraph, with the Fruchterman-Reingold layout with $10^4$ permutations and greedy optimization of modularity algorithm for modularization. The proportion of sensitive OTUs among the total OTUs and the percentage of bacterial taxa in the sensitive OTUs was defined as the “effect size” of sensitive OTUs and sensitive species in the bacterial communities, respectively.

Structural equation modeling (SEM) allows multiple individual linear models to be tested together in a single causal network and evaluates complex causal relationships between variables by converting hypothetical causal relationships into expected statistical patterns[22]. SEM was used to analyze the relative importance of climatic factors, fermentation pH, and microbial composition, and their impact on ARGs. It was assumed that climatic factors had either a direct or indirect effect on fermentation pH and microbial composition in the model. The standardized coefficient of each path in each model component is shown in Fig. 6. Bivariate relationships between all the variables were tested using simple linear regression to ensure that the linear models were appropriate prior to SEM. Chi-square analysis and associated P-values were used to adjust the model (a good fit was defined when $0 \leq \chi^2 \leq 2$ and $0.05 < P \leq 1.00$)[23].

Results
Bacterial communities and succession in corn silage from different climate zones

Bacterial richness was higher in the Heilongjiang and Guizhou provinces and lower in Hainan than other climate zones. The richness and diversity of all silages decreased from days five to 90 except for the Hainan silage (Table S2). After five days of ensiling, the principal component analysis (PCA) plot showed that microbiota from the Hainan and Shanxi silages were separated from other silages and clustered in the first and second quadrants, respectively (Fig. 1A). After 90 days, microbiota from each silage were more dispersed on the PCA plot, however, those from Hainan and Shanxi were still separated from the other silages (Fig. 1B).

Succession of the bacterial community at the genera level that occurred during ensiling of whole-crop corn is shown in Fig. 1C. After five days of ensiling, *Lactobacillus* was the predominant genus in all silages except for the Hainan silage, in which *Leuconostoc* was the most abundant genus (48.6%). After 90 days, all silages were dominated by *Lactobacillus*, and the silage from Hainan also had a relatively high abundance of *Acetobacter*. While *Leuconostoc, Weissella*, and *Klebsiella* were detected in all silages on day five of ensiling, they markedly decreased or disappeared after day 90. After five days of ensiling, *Leuconostoc* was the second most predominant genus, followed by *Klebsiella*, in silages from Xinjiang and Shanxi. More genera with a relative abundance >1% were detected in Tibet silage fermented for five days, which was characterized by a relatively lower abundance of lactic acid bacteria (LAB) than silages from other climate zones.

Succession of the bacterial community at the species level during ensiling of whole-crop corn is illustrated in Fig. 1D. The diversity of the bacterial community decreased with the fermentation process. *Lactobacillus brevis* was observed in all silages except for those from Hainan fermented for five days and those from Guizhou fermented for 90 days. *Lactobacillus plantarum* was detected in all silages except those from Hainan fermented for five days but the abundance decreased until day 90. No *Lactobacillus buchneri* was detected in all five-day silages, but those from Xinjiang, Heilongjiang, Guizhou, and Tibet fermented for 90 days became the dominant species in Xinjiang and Tibet silages. *Lactobacillus paralimentaius* was detected in silages from Xinjiang, Heilongjiang, and Guizhou fermented for five days, and decreased after 90 days of ensiling. *Lactobacillus parafarragins* was only detected in silages from Xinjiang and Guizhou fermented for 90 days. *Lactobacillus paracasei* and *Lactobacillus coryniformis* were detected in silages fermented for 90 days from Shanxi and Guizhou, respectively. Fermentation of the Hainan silages was different from the other silages. After five days of ensiling, *Leuconostoc mesenteroides, Leuconostoc pseudomesenteroides*, and *Lactococcus lactis* were the dominant species in Hainan silages, but by 90 days, they were out competed by *Lactobacillus brevis, Lactobacillus plantarum*, and *Acetobacter fabarum*.

Microbial co-occurrence in corn silage from various climate zones in China

OTUs with significantly different levels of abundance in different climate zones were defined as sensitive OTUs. The distribution patterns of sensitive OTUs in co-occurrence patterns of bacterial communities were assessed in whole-crop corn silages from different climate zones in China (Fig. 2). In silages from
Guizhou, Heilongjiang, Shanxi, Tibet, Xinjiang, and Hainan, fermented for five days, 83, 67, 56, 135, 38, and 33 sensitive OTUs were found, respectively (Additional file 2). These accounted for 4.1, 3.3, 2.8, 6.7, 1.9, and 1.6% of the total community sequences, respectively, according to an approximation for the “effect size” of climate zones on bacterial communities. In silages from Guizhou, Heilongjiang, Shanxi, Tibet, Xinjiang, and Hainan fermented for 90 days had 37, 47, 17, 42, 3, and 25 sensitive OTUs which corresponded to effect sizes of 4.5, 5.7, 2.1, 5.1, 0.4, and 3.0%, respectively. *Lactobacillus* was a sensitive genus in corn fermented for five days from all climate zones except Hainan, accounting for 59.0, 68.7, 8.9, 28.1, and 57.9% of the total sensitive species from Guizhou, Heilongjiang, Shanxi, Tibet, and Xinjiang, respectively, and increasing to 100, 97.9, 70.6, 14.3, and 100% in silages fermented for 90 days. On day five, *Leuconostoc* (33.3%) was the most sensitive species in Hainan silage, evolving to *Lactobacillus* (100%) on day 90. In addition, of the sensitive genera in fermented silage from Tibet, 14.1% *Raoultella* and 8.9% *Enterobacteriaceae* were present at day five, and 16.7% *Enterobacter* were present on day 90.

The abundance of bacterial communities correlated with climate zones. Bacterial communities in silages from different climate zones were clustered into four modules composed of relatively high proportions of sensitive OTUs. After five days of ensiling, module 2 was separated from other modules that primarily contained sensitive OTUs specific to the Tibet zone (Fig. 2A and 2C). After 90 days of fermentation, module 5, module 1, and module 4 were separated from other climate zones that primarily contained sensitive OTUs specific to the Heilongjiang, Tibet, and Hainan regions, respectively (Fig. 2B and 2D). The sensitivity of these module members to various climate zones and their distribution in the network did not match the PCA results. Climate zone modules included a taxonomically broad set of bacteria, indicating differences in the qualitative taxonomic composition of various climate zones were different. After five days of fermentation, the sensitive OTUs in module 4 were mainly composed of *Lactobacillus* and *Weissella*, and *Lactobacillus* was the dominant sensitive genus in module 5, while the sensitive OTUs in modules 1 and 2 were composed of high levels of other microbes, with the exception of LAB (Fig. 2E and 2F). After 90 days of ensiling, the sensitive OTUs in modules 2 and 5 were primarily composed of different *Lactobacillus* species. In module 1, however, *Enterobacter, Acinetobacter, Klebsiella, Raoulteilla,* and other microbes occupied the sensitive OTUs. In module 4, the sensitive OTUs were dominated by *Lactobacillus* and *Acetobacter* (Fig. 2G and 2H).

**ARGs in whole-crop corn silage**

A total of 134 ARGs potentially conferring resistance to 15 classes of antibiotics were detected in whole-crop corn silage (Additional files 3 and 4). Of these, 45 beta-lactamase, 30 multidrug, 15 aminoglycoside, 10 tetracycline, and other classified resistance genes dominated the ARGs (Fig. 3A). Most ARG abundance was impacted by antibiotic efflux, antibiotic inactive, and antibiotic target protection mechanisms (Fig. 3E and 3F). About 17 - 20% of ARGs in corn silage from Guizhou were influenced by antibiotic target alteration mechanisms. In addition, about 40% of ARGs were resistant to antibiotics in corn silage from Shanxi and Hainan by other means.
The PCA indicated that ARG compositions in different climate zones were classified into three clusters (Fig. 3C and 3D). After five days of fermentation, the ARGs in Xinjiang, Heilongjiang and Guizhou silage samples formed one cluster while, the ARGs in Shanxi and Hainan silage samples formed another. The Tibet ARGs were clearly separated from those in samples from other climate zones. After 90 days fermentation, the ARGs distribution in different climate zones was similar to the distribution in silages fermented for five days except for ARGs from Heilongjiang samples, which clustered with Shanxi and Hainan rather than Xinjiang and Guizhou. After five days of ensiling, the abundance of ARGs (absolute abundance of ARGs/detected gene number) in Xinjiang, Guizhou, and Hainan samples, followed by the abundance of ARGs in Heilongjiang and Shanxi samples, were significantly higher than ARGs in other climate zones. The abundance of ARGs from Tibet was lowest (0.65%) (Fig. 3E). The ensiling process dramatically decreased ARG abundance with these exception of samples from Hainan (3.80%). The abundance of Tibet ARGs remained the lowest (0.40%) after 90 days of fermentation (Fig. 3F). The diversity of ARGs in corn silage from Hainan and Shanxi on day five was highest, followed by Xinjiang (Fig. 3G). On day 90 of ensiling, ARG diversity was higher than that seen on day five, except for samples from Shanxi and Tibet (Fig. 3H). In general, the diversity of ARGs in samples from Tibet was lower than regions in other climate zones.

**ARG composition in corn silages from various climate zones**

The top 30 ARGs at five and 90 days of fermentation of whole-crop corn silage from various climate zones is shown in Fig. 4A. Results showed differences in silage ARGs from various climate zones. The dominating ARGs were \textit{tetM}, \textit{oqxB}, \textit{ImrD}, \textit{InuA}, \textit{ermB} and \textit{tetS} in whole-crop corn silage, and the ARGs in Tibet silages were primarily comprised of \textit{ImrD} (45.5-53.1%) and \textit{tetS} (18.8-26.4%). The ARGs in Guizhou silage were mainly composed of \textit{tetM} (28-30%), \textit{InuA} (28-32%), and \textit{ermB} (17-21%), and no difference was observed between silages fermented for five and 90 days. Linear discriminant analysis effect size (LDA-LEfSe) was used to identify ARGs that differed significantly in abundance among five and 90-day silages from various climate zones. The ARG \textit{oqxB} (11.0%) was enriched in silage from Tibet on day five, and enriched in silage from Xinjiang (17.6%) and Heilongjiang (22.7%) on day 90. After fermentation for five days, the ARG \textit{ImrD} from Tibet (53.1%) and Hainan (22.7%), \textit{ANT_6-la} (7%) and \textit{InuA} (19%) from Xinjiang, \textit{Klebsiella pneumoniae acrA} (11.2%) from Xinjiang, and \textit{tetS} (0.61%), \textit{ermB} (13.0%), \textit{InuA} (21.7%) and \textit{tetM} (48.2%) from Heilongjiang were particularly enriched. The ARG \textit{LEN-25} (4.1%) and \textit{poxtA} (8.9%) and \textit{tetS} (26.4%) from Shanxi and Tibet, respectively, were particularly enriched in corn silage on day 90.

The ARGs conferring resistance to 25 different drug classes were detected in corn silages from various climate zones (Fig. 4B and Additional file 5). The ARGs detected in silages from various climate zones mainly corresponded to the drug classes tetracycline, lincosamide, fluoroquinolone, glycyclycline, macrolide, diaminopyrimidine, nitrofuran, cephalosporin, penam and streptogramin. The composition of drug classes conferred by ARGs in Shanxi and Hainan silages were very similar, and no difference was observed between the early and late fermentation stages. The ARGs belonging to the drug classes tetracycline and lincosamide accounted for large proportions of the total drug classes in silages from Guizhou and Tibet. The ARGs corresponding to the drug classes, fluoroquinolone, glycyclycline, and
diaminopyrimidine were enriched to a greater extent in the late fermentation stage of Xinjiang and Heilongjiang silages. The ARGs from the lincosamide and macrolide classes were enriched in early fermentation stage of the Xinjiang, Heilongjiang and Guizhou silages. Cephalosporin, and penam were the significantly enriched drug classes in the late fermentation stage of Heilongjiang silage. Fluoroquinolone, glycylcycline, and diaminopyrimidine resistance genes were enriched in the early fermentation stage and phenicol resistance gene was specifically enriched in the late fermentation stage of Tibet silage.

**The effect of climate factors, pH, and microbial composition on ARGs from corn silage**

To access the effect of climate factors, silage fermentation pH, and microbial composition on ARGs, correlation analysis was conducted with SEM. The first principal component of microbial composition was extracted as the microbial constituent elements for SEM analysis. The first principal component of the microbial composition explained 53.2% of the total variation, so could reliably describe the contribution of the microbial composition in SEM. The suitability of climate factors, silage fermentation pH and silage microbial composition to the ARG action model is $\chi^2 = 0.045$, df = 1, and $P = 0.832$ (Fig. 5). The results indicated that precipitation, sunshine duration, and mean relative humidity had indirect effects on ARGs. Profiling of the microbial composition indicated that precipitation and pH had an indirect effect on ARGs, and the sum of these variables could explain 55% of the variation. Of the climate factors, mean temperature and precipitation had a positive effect, fermentation pH had a negative effect, and microbial composition had a direct positive effect on ARGs. SEM confirmed that microbial composition was the most important driver of the resistance gene (Table S5).

**Identification of ARG hosts in corn silage from different climate zones**

The hosts of major annotated ARGs in corn silage differed between climate zones (Fig. 6). *Enterobacter*, *Klebsiella*, *Staphylococcus*, *Lactobacillus*, and *Lactococcus* were primary hosts of ARGs in silages from a variety of climate zones. *Enterobacter* and *Klebsiella* were the primary hosts of *oqxB*, *oqxA* and *Klebsiella pneumoniae acrA* in all silages except for Guizhou silage. *Enterobacter* and *Klebsiella* were primary hosts of *Klebsiella pneumoniae KpnG*, *Klebsiella pneumoniae KpnE* and *Klebsiella pneumoniae Ompk 37* in Shanxi, Heilongjiang, Tibet, and Hainan silages. The ARGs of *tetM*, *ermB* and *InuA* were primarily from *Staphylococcus* and *Lactobacillus* in Xinjiang, Heilongjiang, and Guizhou silages while *Lactobacillus* in Tibet silage only carried *InuA*. The *ImrD* was carried by *Lactococcus* in Xinjiang, Tibet, Shanxi and Hainan silage. In Tibet silage, the LAB *Enterococcus* carried *tetS*, *tetM* and *catA8*, while *Lactococcus* and *Streptococcus* carried *tetS* and *tetL*, respectively.

**High-risk ARGs in whole-crop corn silages from different climate zones**

The World Health Organization (WHO) and literature review identified 73 high-risk ARGs (of these, 37 were proposed by WHO). These ARGs have the highest potential to public health and were named “current threats”. Some ARGs that could transfer to pathogens were found in gut microbiota like *Bacteroides* and *Lactobacillus* or their close relatives, and were named “future threats”. In the present study, eight “current
threat” ARGs (\textit{tetM}, \textit{ermB}, \textit{SHV-1}, \textit{CTX-M-2}, \textit{dfrA17}, \textit{lnuA}, \textit{tetL} and \textit{floR}) and three “future threat” ARGs (\textit{vatE}, \textit{tetO} and \textit{tetW}) were found in silage from different climate zones in China (Fig. 7).

\textit{TetM}, \textit{ermB} and \textit{lunA} were the most abundant high-risk ARGs in Guizhou, Heilongjiang, and Xinjiang silage, and the fermentation process decreased the relative abundances of the three ARGs. \textit{ErmB} and \textit{lunA} were also observed in Tibet silages with relative abundances of 0.6 - 1.2% and 1.7 - 1.9%, respectively. \textit{SHV-1} was present in silage from Xinjiang, and increased from 0.78% on day five to 2.88% on day 90 of fermentation. The ARG \textit{CTX-M-2}, \textit{dfrA17}, and \textit{floR} were not tested in silage fermented for five days, but were found in silage on day 90, with the highest abundance from Heilongjiang (0.29%, 0.26%, and 0.27%) and Guizhou (0.09%, 0.04%, and 0.1%). Corn silage from Tibet had the highest high-risk ARG of \textit{tetL} with relative abundances of 1.3% and 1.6% in corn after five and 90 days of ensiling, respectively. The relative abundances of the “feature threat” ARGs, \textit{vatE} and \textit{tetW}, in Guizhou silage (0.6% and 0.2% on day five and 90, respectively) were highest, followed by those from Heilongjiang (0.2% and 0.08% on day five; 0.02% and 0.00 on day 90). \textit{TetO} presented on day five with relative abundances of 0.4%, 0.2%, and 0.007% in corn silage from Heilongjiang, Guizhou, and Xinjiang, respectively.

\textbf{Discussion}

The richness and diversity of corn silage bacterial communities from different climate zones varied greatly. Epiphytic microbiota in fresh forage is one of the important factors affecting the richness and diversity of bacterial communities in ensiled forage\cite{24}. The structure of epiphytic microbial communities depends on many factors including plant species, vegetation period, geographic location, climate, solar radiation intensity, and fertilizer type\cite{25–27}. Some of these factors influencing the micro-ecological process of plant surfaces likely contribute to differences in microbial community composition and the succession of corn silages from different climate zones. Results from this study showed differences in the bacterial composition of silage from different climate zones that were fermented for five or 90 days. While \textit{Lactobacillus} was the predominant genus in all silage except for silage from Hainan fermented for five days, the bacterial composition at species level remained entirely different. Keshri et al.\cite{28} reported that \textit{Lactobacillus} spp. became the dominant genus after two days of ensiling, but was found at lower abundance in fresh corn. The prevalence of \textit{Leuconostoc} in five-day Hainan silage was probably caused by the unique climate. The Hainan silage had a higher pH than other silages after five days of ensiling (4.27 vs. <3.89, respectively; Table S4), indicating that fermentation of Hainan silage was retarded. In general, the relative abundance of \textit{Leuconostoc}, \textit{Weissella}, and \textit{Klebsiella} in silage markedly decreased from days five to 90. Both \textit{Leuconostoc} and \textit{Weissella} are heterofermentative LAB in the \textit{Leuconostocaceae} family that are associated with the early stage of fermentation and are gradually replaced by \textit{Lactobacillaceae} during ensiling\cite{29,30}. A pH <4.0 inhibits the growth capability of \textit{Klebsiella}\cite{31}, decreasing this bacteria to <5% after 90 days of ensiling, which is consistent with the present study.

Ensiling fermentation is initiated by homofermentative strains and gradually replaced by heterofermentative species like \textit{Lb. buchneri} and \textit{Lb. brevis}\cite{32}. In the present study, \textit{Lb. brevis} was
detected in all silage and become the most dominant species in Shanxi and Hainan silages on day 90, while *Lb. buchneri* became the predominant species in Xinjiang, Guizhou, and Tibet silages after 90 days of fermentation. *Lactobacillus parafarraginis*, in the *Lb. buchneri* group, produce acetic acid and Xu et al. [33] reported that *Lb. brevis* dominated corn stover silage before day 20 and later shifted to *Lb. parafarraginis*. This study showed an increase in the relative abundance of *Lb. parafarraginis* in Xinjiang and Guizhou silages after 90 days of ensiling. About 25% of *Acetobacter fabarum* occurred in day 90 corn silage from Hainan. For forage ensiling, *Acetobacter* was a genus of undesirable bacteria that is commonly found in corn silage and can initiate aerobic deterioration[34].

In early and late fermentation stage meta-networks, sensitive OTUs were grouped in variable modules that reflected the different climate zones. Large groups of bacteria were found to respond similarly to specific climate zones and clustered together in networks. The sensitive genus in the early and late stages of fermented corn is associated with bacterial community structure. *Lactobacillus* was the most prevalent sensitive bacteria on days five and 90 except for Hainan silage at day five, in which *Leuconostoc* was the most sensitive genus, indicating that, with the exception of Hainan silage, the rapid fermentation stage of corn silage was complete in the first five days. *Enterobacteriaceae* was the most sensitive genus in Tibet silage, suggesting that low pH and silage end products could not inhibit these bacteria. This is likely because these microorganisms developed an ability to resist the extreme environment, including low temperature, hypoxia, strong ultraviolet radiation, low accumulated temperature, and low air pressure, observed in Tibet[35].

The high diversity of the resistome in corn silage from different climate zones in China suggests that ARGs are universal and present in a wide range of ecosystems. The ARGs detected in corn silage include those that can potentially provide resistance to major antibiotics used to treat animals and humans, including multidrug (*sme, ade, acr* and *Klebsiella pneumoniae* et al. genes), beta-lactams (*OXY, LEN, ACT, CTX, CMY, OXA* et al. genes), aminoglycosides (*ANT, APH, AAC, aad* and *sme* genes), tetracyclines (*tet* genes), fluoroquinolones (*oqx* and *qnr* genes) and lincosamides (*Imr* and *Inu* genes). Wu et al.[15] reported that tetracycline and macrolide antibiotic genes were dominant in corn kernel silage. It is not surprising that ARGs exist in silage because pathogenic bacteria is commonly associated with water, soil, and plants[36]. In addition, antimicrobial-resistant bacteria and ARGs enter the food chain through groundwater and animal manure that is spread to agricultural plants[37]. Results in the current study showed that the variance in bacterial community composition led to remarkable differences in ARG diversity and composition in corn silages from different climate zones. The fermentation process decreased the relative abundance of ARGs except Hainan silage because the oxygen and pH reduction that occurs during ensiling can inhibit pathogen growth[38]. The higher abundance of ARGs in Guizhou, Xinjiang and Hainan silages on day five might be the result of the higher temperatures in these regions[39,40] (Table S2). The increase in richness and diversity of the bacterial community increased ARG abundance in Hainan silage between days five and 90. The higher relative abundance of *Acinetobacter* in silage on day 90, and some *Acinetobacter* ARGs (*ade, ADC* and *MCR* genes) may contribute to the increase in ARGs. The diversity of ARGs in corn silage was higher on day 90 than five
except for silage from Tibet and Shanxi. This may be because plasmids are the platforms on which ARGs are assembled and reasserted[41], and a resistance plasmid is able to carry one or more ARGs[42].

ARG composition in different climate zones varied by climate condition and plant surface microenvironments. The ARGs in silage from warm and humid climate zone (Guizhou; Cfa) was primarily comprised of *tetM*, *InuA* and *ermB*, while *tetM*, *oqx*B, *ImrD* and *InuA* were the predominant ARGs in silage from the arid climate zone (Xinjiang; BWk). The ARGs in silage from relative arid climate zones (Shanxi; BSk and Hainan; Aw) were mainly composed of *oqx*B, *Klebsiella pneumoniae KpnG*, *Klebsiella pneumoniae acrA*, and *oqxA*. Silage in relatively high humidity but low temperature climate zone (Heilongjiang; Dwa) was dominated by *tetM*, *InuA*, *ermB* and *oqx*B ARGs. The diversity (mainly containing *ImrD* and *tetS*) and the total number of ARGs in Tibet (Dwc) silage was lower than silage from other climate zones, indicating that antibiotic contamination in Tibet is relatively low. Besides the more commonly used antibiotics like lincosamide, tetracycline, fluoroquinolone, macrolide and streptogramin, Tibetan corn silage conferred relatively higher resistance to phenicol, pleuromutilin, and oxazolidinone, which are commonly used in livestock[43–45]. Local climate conditions and spatial distances significantly influenced microbial community and functional genes distribution[39,46]. Previous studies showed that the abundance of ARGs in pristine Tibet soil (without human impact) is not necessarily reduced, but ARG types are considerably different from those found in environments impacted by humans[47], and human activity is thought to influence the acquisition of antibiotic resistance in bacteria[48]. The major method of animal husbandry in Tibet is grazing, thus, the intensity of human activity is significantly lower than that found in intensive production environments. As a result, it is possible that special environmental factor, low intensity agricultural measures, and animal husbandry management practices shaped the lower diversity and abundance of ARGs found in Tibet than other climate zones.

Antibiotic efflux, antibiotic inactive and antibiotic target protection mechanisms contributed to most of the ARG abundance in corn silage. Most multidrug resistance in microorganisms is dependent on efflux pumps[49], however, there is no correlation between the number of efflux pumps and the phenotype of antibiotic resistance. Efflux pumps may help bacteria to adapt to diverse environments[50]. Thus, ARGs that resist antibiotics via efflux pumps may be better adapted to different ecosystems and more easily able to transfer horizontally between bacterial hosts. Combined with the results of ARG abundance and ARG hosts identification, major ARG hosts in corn silage from different climate zones were identified. The major ARG hosts in Xinjiang and Heilongjiang corn silages were *Lactobacillus* and *Staphylococcus*, and *Enterobacter* also be the major host in Heilongjiang silage. The major hosts of ARGs in Shanxi and Hainan corn silage were *Enterobacter* and *Klebsiella*, while the major hosts of ARGs in Guizhou and Tibet corn silage were *Lactobacillus* and *Lactococcus*, respectively. *Enterobacteriaceae* and *Staphylococcus* are common pathogens that are significantly suppressed during ensiling. *Lactobacillus* and *Lactococcus* are generally recognized as safe, and some strains of LAB confer a health benefit on humans and animals[51,52]. However, antibiotic resistance of LAB from food, including dairy and fermented products is focused. Some LAB species have antibiotic resistance and the ARGs are shown to transfer between different species[53,54]. *Lactobacillus* carries the *vanX*, *vanE*, *gyrA*, *tetM* genes[55] in
dairy products, and vanZ and cat genes in fermented foods[56]. Lactobacillus plantarum and Lb. reuteri contain tetW and erm genes[57]. The resistance genes, lmr, gyr, tet, van are also detected in Lactobacillus and the prophage may be a critical vector for Lactobacillus ARGs[58]. In the present study, however, van and gyr genes were not detected. In general, the potential risk of ARGs in Xinjiang, Guizhou, and Heilongjiang silage appears to be derived largely from Lactobacillus, but this requires future verification. While silage safety is the basic insurance for steady livestock development, less attention is given to the antibiotic resistance of silage. Inoculants are widely used to improve fermentation quality during silage production but antibiotic resistance and inoculant safety have received little attention. As a result, safety should be assessed before silage inoculants are applied in the future.

The spread of ARGs is considered a great threat to human health. As a result, this study focused on 11 ARGs identified as high-risk resistance genes to human health. Eight “current threat” ARGs[59,60], tetM, ermB, SHV-1, CTX-M2, dfrA17, lnuA, tetL, and floR were detected, with the most abundant, tetM, ermB and lnuA, found in Guizhou, Heilongjiang, and Xinjiang corn silage. These three ARGs were shared across non-pathogens such as Lactobacillus and pathogens like Staphylococcus. The “current threat” ARGs have a wide host range and niche adaptation. These “current threats” were either transmitted from non-pathogens to pathogens or originated from pathogens[60,61]. Additional studies into the safety of silage and silage inoculants can provide guidance on proactive strategies to reduce the spread of ARGs into animal products. The vatE, tetO, and tetW identified as “future threats”[60] were discovered in Guizhou and Heilongjiang corn silages. The relative abundance of “current threats” and “future threats” in 90-day silage was lower than that seen in five-day silage, except for tetL gene from Tibet silage. This suggest that the fresh corn planted in these regions was impacted by human practices and improved the spread of ARGs from agronomic measures to livestock. In addition, feeding silage could decrease ARG spread more than fresh forage. The ARG tetL in Tibet silage (1.3% on day five and 1.6% on day 90) was linked to Streptococcus with low relative abundance (0.02%; including 0.007% of Streptococcus cristatus, 0.007% of Streptococcus anginosus, and 0.007% of unclassified Streptococcus). Gao et al.[62] reported that the most frequent tet gene was tetL from Streptococcusagalactiae in cows with mastitis. This may be explained the manure of cows with mastitis was used as farmyard manure to plant corn in Tibet, resulting in the spread of ARG tetL. Antimicrobial resistance and ARGs in silage from the environment and additives requires urgent monitoring.

Conclusion

Bacterial communities and ARG reservoirs in corn silage differ across climate zones. Bacterial communities respond to climate zones and are dominated by the genus Lactobacillus in all climate zones. Differences in the resistome among climate zones was primarily mobilized by bacterial community composition. The ensiling process reduced ARG abundance. The Tibet corn silage had remarkably lower diversity and abundance of ARGs compared with silages from other climate zones. The resistome in silage from Cfa, Dwa and BWk climate zones was characterized by ARGs that conferred a high risk to human health. We recommend that more attention need to be paid to AGRs and their mobile
genetic elements and hosts in silage. This will help to decrease risk and control ARG spread from agriculture and livestock to humans.

**Abbreviations**

AMR: antimicrobial resistance

ARG: antibiotic resistance genes

ARB: antimicrobial resistant bacteria

SMRT: single-molecule real-time sequencing

CARD: Comprehensive Antibiotic Resistance Database

LRT: likelihood ratio tests

SEM: structural equation modelling

PCA: principal component analysis

LDA-LEfSe: linear discriminant analysis effect size

WHO: World Health Organization

LAB: lactic acid bacteria

**Declarations**

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**Contributions**

XSG, DMX, XJY, HYH and YLX designed the experiment. HYH, CW, WKH, LEG, FHL, YXZ, JH, JW, YBL, FW, LFL and JYL collected the samples. DMX, NN, HYH, CW and RRS conducted the experiment. DMX analyzed the data and wrote the manuscript. DM guided the bioinformatic analyses. DMX, XJY, HYH and XSG provided advice and revised this manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All sequence data used in the study is available at National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under accession numbers PRJNA782218 and PRJNA783360.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


27. McGarvey JA, Franco RB, Palumbo JD, Hnasko R, Stanker L, Mitloehner FM. Bacterial population
dynamics during the ensiling of medicago sativa (alfalfa) and subsequent exposure to air. J Appl
dynamics of the bacterial communities developed in maize silage. Microb Biotechnol.
2017;10:1663–76.
30. Silva R, Costa DM, Santos EM, Moyer K, Hellings E, Kung L. The effects of Lactobacillus hilgardii
4785 and Lactobacillus buchneri 40788 on the microbiome, fermentation, and aerobic stability of
33. Xu Z, He H, Zhang S, Kong J. Effects of inoculants Lactobacillus brevis and Lactobacillus
paraffarraginis on the fermentation characteristics and microbial communities of corn stover silage.
34. Spoelstra SF, Courtin MG, Van B. Acetic acid bacteria can initiate aerobic deterioration of whole crop
35. Rampelotto PH. Resistance of microorganisms to extreme environmental conditions and its
36. Tope AM, Hitter AC, Patel S V. Evaluation of antimicrobial resistance in enterobacteriaceae and
coliforms isolated on farm, packaged and loose vegetables in kentucky. J Food Microbiol Saf Hyg.
2016;01:1–7.
37. Muhammad J, Khan S, Jian Q, Hesham EL, Gogoi B. Antibiotics in poultry manure and their
38. Queiroz O, Ogunade IM, Weinberg Z, Adesogan AT. Silage review: Foodborne pathogens in silage and
and abundance of antibiotic resistance genes in wetlands on the Qinghai-Tibetan Plateau. J Hazard
Mater. 2019;361:283–93.
40. Novo A, André S, Viana P, Nunes OC, Manaia CM. Antibiotic resistance, antimicrobial residues and


59. WHO. 2019 Antibacterial agents in clinical development an analysis of the antibacterial clinical development pipeline.


Figures
Figure 1

Bacterial community dissimilarities of corn silage. (A, B) PCA analysis showing dissimilarities of bacterial communities in silages fermented for five and 90 days among different climate zones. (C, D) Relative abundances of bacteria at genus and species level (Top 30) of silages ensiled for five and 90 days across different climate zones. X, Xinjiang province; S, Shanxi province; H, Heilongjiang province; G, Guizhou province; T, Tibet province; K, Hainan province.
Figure 2

Co-occurrence patterns of sensitive OTUs in different climate zones. (A, B) Co-occurrence networks visualizing significant correlations ($p > 0.7$, $P < 0.001$; indicated with gray lines) between bacteria OTUs in corn silage communities (ensiled for five and 90 days). OTUs are colored by their association to the different climate zones (gray OTUs are insensitive to cropping practices). Shaded areas represent the network modules containing sensitive OTUs. “Y_Z” represent the OUT sensitive to “Y” and “Z” climate zone. (C, D) Cumulative relative abundance (as counts per million, CPM; y-axis in $\times 1000$) of all bacteria of the sensitive modules in networks of bacterial communities in silage ensiled for five and 90 days. The cumulative relative abundance in samples of X (Xinjiang province); S (Shanxi province); H (Heilongjiang province); G (Guizhou province); T (Tibet province) and K (Hainan province) indicates the overall response of sensitive modules to the different climate zones. (E-H) Qualitative taxonomic composition of sensitive modules is reported as proportional OTUs numbers per genus (bacteria) and species and compared to the overall taxonomic distribution in the entire dataset (column “all”).
Figure 3

Composition of ARGs in silage ensiled for five and 90 days. (A) Antibiotic resistance gene classification. (B) Resistance mechanism. (C, D) PCA analysis showing dissimilarities of ARGs in silages fermented for five and 90 days from different climate zones. (E, F) Relative abundances of ARGs (absolute abundance of ARGs/detected total gene number) in silages fermented for five and 90 days from different climate zones. (G, H) Diversity of ARGs (Shannon index) in silages fermented for five and 90 days from different climate zones.
climate zones. X, Xinjiang province; S, Shanxi province; H, Heilongjiang province; G, Guizhou province; T, Tibet province; K, Hainan province.

Figure 4

Relative abundance of ARGs and comparison between early (five day) and later (90 day) stage of silage fermentation among different climate zones. (A) top 30 abundance of ARGs and linear discriminant...
analysis (LDA) effect size (LEfSE) analysis among ensiling process. Histogram of ARGs that differentiate with statistical significance, ordered by effect size. (B) ARG abundance of different antibiotic resistance and LEfSE analysis among ensiling process. Histogram of antibiotic resistance that differentiate with statistical significance, ordered by effect size. X, Xinjiang province; S, Shanxi province; H, Heilongjiang province; G, Guizhou province; T, Tibet province; K, Hainan province.

Figure 5
Structure equation models of climatic factors during the corn growth period, silage fermentation pH and microbial composition as predictors of ARGs. Solid arrow indicates the path direction and the effect is significant ($P<0.05$), and the dashed arrow indicates the path direction and the effect is not significant ($P>0.05$). The number adjacent to the arrow is the normalized path coefficient. $R^2$ and $\chi^2$ represent the model variance explanation scale and model fitness, respectively. PRE, precipitation; MT, mean temperature; MRH, mean relative humidity; SD, sunshine duration; Micr, silage microbial composition; ARGs, antibiotic resistance gene; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

**Figure 6**

ARGs and the corresponding bacteria using a Circos plot. Left and right hemi circle represents the bacteria and ARG names, respectively. The length of the main circular segments is proportional to the total number of ARGs belonging to that segment, while the width of the ribbon connecting the gene with the bacteria represents the proportion of ARGs belonging to the particular bacteria. The two outer rings are contribution tracks, *i.e.*, stacked bar plots with a gradient of color signifying the proportion of entries from different genes. X, Xinjiang province; S, Shanxi province; H, Heilongjiang province; G, Guizhou province; T, Tibet province; K, Hainan province.

**Figure 7**

Relative abundance of 11 high risk ARGs in silages on day five and 90 from different climate zones. Different letters are significantly different ($P<0.05$). Bars are standard errors. X, Xinjiang province; S, Shanxi province; H, Heilongjiang province; G, Guizhou province; T, Tibet province; K, Hainan province.

**Supplementary Files**

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