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Phyllosphere antibiotic resistome in a natural primary vegetation across a successional sequence after glacier retreat

Running title： Phyllosphere antibiotic resistome in a vegetation successional sequence

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Highlights

- The ARG abundance decreased during the plant community succession process.

- There was a core set of the phyllosphere ARGs in plant community succession process.
- Leaf property and surrounding habitat result in varied phyllosphere ARG abundance.
- The phyllosphere ARG profile is linked to the leaf bacteria and nutrient content.

Abstract

The spread of antibiotic-resistance genes (ARGs) has posed a significant threat to human health over the past decades. Despite the fact that the phyllosphere represents a crucial pool of microorganisms, little is known about the profile and drivers of ARGs in less human interference natural habitats. In order to minimize the influence of environmental factors, here we collected leaf samples from the early-, middle- and late-successional stages across a primary vegetation successional sequence within 2 km, to investigate how the phyllosphere ARGs develop in natural habitats. Phyllosphere ARGs were determined using high-throughput quantitative PCR. Bacterial community and leaf nutrient content were also measured to assess their contribution to the phyllosphere ARGs. A total of 151 unique ARGs were identified, covering almost all recognized major antibiotic classes. We further found that there was some stochastic and a core set of the phyllosphere ARGs during the plant community succession process, due to the fluctuant phyllosphere habitat and specific selection effect of plant individuals. The ARG abundance significantly decreased due to the reduction of the phyllosphere bacterial diversity, community complexity, and leaf nutrient content during the plant community succession process. While the closer links between soil and fallen leaf resulted in a higher ARG abundance in leaf litter than in fresh leaf. In summary, our study reveals that the phyllosphere harbors a broad spectrum of ARGs in

the natural environment. These phyllosphere ARGs are driven by various environmental factors, including the plant community composition, host leaf properties, and the phyllosphere microbiome.

Keywords

Antibiotic resistome, bacterial community, fresh leaf, leaf litter, leaf nutrient, plant community

1. Introduction

Antibiotic resistance is a growing threat to human health and the clinical treatment of diseases worldwide (Hernando-Amado et al. 2019; Zhang et al. 2022b). Over recent decades, with the widespread clinical use of antibiotics, associated resistance genes (ARGs) have spread rapidly and can be detected in almost all ecosystems (Martinez et al. 2015; Zhang et al. 2022b; Zhu et al. 2017). So far, most studies have been focused on agricultural and urban ecosystems (Pärnänen et al. 2019; Yu et al. 2022; Zhu et al. 2021a). In contrast, the distribution of ARGs in natural environments remains poorly characterized.

The plant-associated microbial pool is considered a critical terrestrial microbial reservoir harboring a high diversity of microorganisms (Vorholt 2012; Xu et al. 2022b; Zhu et al. 2021b). Whilst previous studies have focused on the plant's general microbial community compositions and their ecological functions (Kembel et al. 2014; Meyer et al. 2022), it should also be noted that the plant is a potential reservoir of the antibiotic-resistance microbiome (Hernando-Amado et al. 2019; Martinez et al. 2015). Most

plant-related ARG studies have been conducted in soil and the plant rhizosphere, providing valuable insights into the ARG profile and its potential spread (Chen et al. 2018; Guo et al. 2021), with less attention paid to the phyllosphere-associated ARGs, which may represent a critical source of ARGs.

The leaf surface, or phylloplane, represents one of the largest microbial pools on earth because of the enormous global leaf surface area (Lindow and Brandl 2003; Redford et al. 2010). The phyllosphere is a relatively open habitat that can continuously exchange microbiomes with other habitats (Meyer et al. 2022; Zhang et al. 2022b). There is evidence that the antibiotic-resistant bacteria (ARB) of the phyllosphere originate from the soil and air (Chen et al. 2017; Zhang et al. 2022b). Mechanisms for each of these routes are clear; for example, ARB could reach the leaf surface from the soil by soil-associated aerosols or deposit on the leaf surface by rainfall after entering the atmosphere (Zhang et al. 2022b; Zhu et al. 2021a). These likely colonization routes lead to the argument that the local dispersal of the microbial community is an important determinant of the phyllosphere microbiome (Meyer et al. 2022).

During plant community development, changes in plant physiology or species directly impinge on leaf functional traits driving variation in the associated phyllosphere microbial communities (Hacquard et al. 2017; Kembel et al. 2014; Peng et al. 2021; Xu et al. 2022a). For instance, Kembel et al. (2014) reported that the phyllosphere microbial community varied among tree species and significantly correlated with leaf

nutrient content. Further, plant community structure and associated ecosystem stability increase over the succession of vegetation communities to climax communities (Buma et al. 2017; Mori et al. 2017), but the effects of this on phyllosphere antibiotic resistance remain unknown. In addition, changes in the leaves themselves could also have dramatic effects on their microbiome (Kembel et al. 2014; Lajoie et al. 2020). For instance, the leaf-associated microbiome could also vary due to the change in the characteristics of the leaf itself (such as physiology and nutrient status) after leaf fall (Norby and Cotrufo 1998; Zheng et al. 2021). However, it remains unclear how changes in host leaves nutrients content and other leaf propertities affect the phyllosphere-associated ARB and antibiotic resistome.

The limited number of previous studies focusing on the phyllosphere microbiome and antibiotic resistome have been confined to investigations from broad-scale habitats or controlled growth studies (Martinez et al. 2015; Yan et al. 2021; Zhang et al. 2022b). However, the interpretations from such studies are complicated by either the large variation of the natural environmental factors, including rainfall, temperature, and illumination across large distances, or the absence of variation under controlled growth (Li et al. 2023; Xu et al. 2022b). We, therefore, collected samples from a retreating glacier forefield with a complete primary succession sequence from plant pioneer to climax communities across a 2km distance. This continuous succession in a limited distance reduces the impact of other environmental factors on the succession process and makes this sequence ideal for research into the succession of microbial and plant

communities. We analyzed the phyllosphere ARGs, bacterial communities, and leaf nutrient content aiming to 1) characterize the abundance and profile of the phyllosphere ARGs across this natural primary vegetation succession; 2) evaluate the effect of the plant community successional development and leaf fall on these ARGs and; 3) explore the potential drivers shaping the phyllosphere ARG abundance and profile.

2. Materials and methods

2.1 Study area and sampling

We sampled across a primary succession in the forefield of the retreating Hailuogou Glacier. Detailed information about the sampling location can be found in Lei et al. (2015). Briefly, the Hailuogou Glacier is a monsoonal temperate glacier at the Gongga Mountain (29° 34′ 07.83″N, 101° 59′40.74″ E, 7556 m a.s.l.) on the southeastern edge of the Tibetan Plateau in Sichuan province, China. The mean annual precipitation is 2000 mm, and the mean annual air temperature is 3.8 °C. Glacier recession began at the end of the Little Ice Age (~ 1850) and has accelerated since the early 20th century (Lei et al. 2015). The mild and humid climate of Gongga Mountain enables the rapid growth of vegetation communities and fast development of the ecosystem, resulting in a complete primary vegetation successional sequence from bare ground to climax vegetation community within the 2 km transect sampled (Jiang et al. 2018b). This reduces the impact of other variables on the succession process and makes this sequence ideal for research into the succession of microbial and plant communities.

Leaf samples were collected from five sites, each with four replicates, across the vegetation successional sequence with detailed descriptions of both sites and plant communities summarised in Table S1. The age of each site was calibrated with chronologies according to tree rings and soil erosion rates assessed by the ^{137}Cs method, with sites representing 25, 40, 70, 100, and 120 years, respectively, spanning early (25 and 40 years), mid (70 years) and late (100 and 120 years) successional stages in this system as suggested in previous studies (Jiang et al. 2018a; Jiang et al. 2019) At each site, composite leaf samples were collected from: leaf litter taken from the ground surface (LL), and mature and intact leaves taken from tree and ground cover vegetation, with these two sets of samples collectively referred to as fresh leaf (FL).

Four square plots (10 m × 10 m) were established at each site with a minimum 10-m distance between each plot. FL samples were randomly taken from different individual plants in each plot. A similar mass of leaves was collected for all species with all leaves subsequently mixed to form a single composite sample for each plot. LL samples were collected from beneath the canopy of the FL in each plot. Samples were transported to the lab on ice and processed on the same day. Each sample was divided into two equal aliquots for the following analyses. One aliquot was stored at 4 ℃ and phyllosphere microbial DNA was extracted within one week. The other aliquot was freeze-dried and ground for nutrient determination. Leaf carbon (C), nitrogen (N), and sulfur (S) content were measured by an elemental analyzer (NCS 2500, Carlo Erba Instruments, Milan, Italy). Inductively coupled plasma–optical emission spectrometric (ICP-OES) was used

to determine leaf phosphorus (P) and potassium (K) content (Kalogiouri et al. 2022). Details of the leaf nutrient contents were summarised in Table 1 and Fig. S1.

2.2 DNA extraction

The phyllosphere microbial genomic DNA was extracted using a FastDNA® Spin Kit for Soil (M.P. Biomedical, Santa Ana, California, U.S.A.) after washing leaves according to previous studies (Chen et al. 2020; Zhou et al. 2021). Briefly, ~10 g of leaf material was weighed into a 250 mL conical flask with 100 mL 0.01 M sterilized phosphate-buffered saline (PBS), shaken (180 rpm) for one hour at room temperature (~ 20 ℃), and then passed through a 0.22 μm filter membrane (Diameter =55mm). The membranes were subsequently cut with sterile scissors into small pieces, and all the pieces were used for Genomic DNA extraction according to the manufacturer's instructions. DNA quality was measured by a spectrophotometer (NanoDrop ND-1000, Thermo Scientific, Waltham, MA) and gel electrophoresis and stored at -20 ℃ prior to downstream analysis.

2.3 High-throughput quantitative PCR (HT-qPCR)

High-throughput qPCR was performed using a SmartChip Real-time PCR system (Wafergen, Fremont, CA) to determine the abundance of ARGs following Chen et al. (2017) with primers targeting ARGs, mobile genetic elements (MGEs), and the 16S rRNA gene (Table S2). The data was automatically generated by Wafergen software (based on the CT values normalized with 16S rRNA) and all the melt curve analysis of PCR were checked with single peak. Three technical replicates were assessed per

sample with the detection threshold cycle (C.T.) for successful amplification set to 31. For each primer set, a non-template negative control was included to detect lab contamination. The PCR amplification program had an initial enzyme activation at 95 ℃ for 10 min, 40 cycles of denaturation at 95 ℃ for 30 s, and annealing at 60 ℃ for 30 s. Melting curves were automatically generated by the WaferGen software. Results with multiple melting peaks or with the amplification efficiencies out the range (1.8−2.2) were removed. The relative copy number of each ARG was calculated according to: relative copy number = $10^{(31-CT)}$/ (10/3), where CT represents the threshold cycle and expressed as copies per 16S rRNA gene copy (Schmittgen and Livak 2008).

2.4 Bacterial gene sequencing

Bacterial 16S rRNA was amplified with primers F515 and R907 (Zhou et al. 2011). Amplicons were verified by gel electrophoresis and purified using the Wizard S.V. Gel and PCR Clean-Up System (TIANGEN Biotech, Beijing, China). The concentration of PCR products was quantified by a Qubit 3.0 Fluorometer. Sequencing was performed on an Illumina Miseq platform (Illumina, San Diego, U.S.A.), at the Majorbio Bio-Pharm Technology Co., Ltd Shanghai, China. Established sequence analysis was applied, briefly, FLASH2 software was used to pair and splice sequences before primer sequences were removed in Mothur. Sequences with a total base error rate greater than two and sequences shorter than 100 bp were removed using USEARCH in the QIIME

platform. After denoising in DADA2 amplicon sequence variants (ASVs) were generated, followed by the removal of chloroplast and mitochondrial sequences, and sequences were rarefied to the lowest number of sequences per sample using rarefaction curves to ensure sufficient sequences were present in all samples. Raw reads have been deposited in the NCBI Short Read Archive database under accession number PRJNA838795.

2.5 Statistical analysis

Microsoft Excel was used for the mathematical calculations of the raw data. ARGs were separated into the core, common and stochastic ARGs based on their frequency in the samples, as described in Su et al. (2017) and Chen et al. (2020). ARGs were defined as common and stochastic when detected in greater or fewer than 50% of samples, respectively, with ARGs detected in more than 80% of samples defined as core ARGs. Differences in leaf nutrient content and ARG between samples were identified by ANOVA with posthoc Tukey tests where appropriate, with $P < 0.05$ considered statistically significant. Kruskal-Wallis with multiple check correction (FDR) was used to test the variation of different ARG subgroups and bacterial communities. Microbial co-occurrence network analysis was used to test the community complexity. These analyses were performed using the online tool of Majorbio Cloud Platform (https://cloud.majorbio.com/page/tools/). The profiles of ARG were compared by using principal coordinate analysis (PCoA), and analysis of similarities (ANOSIM) was used to identify the differences in ARG profiles and bacterial community with package

"labdsv" and "vegan" (Oksanen et al. 2019; Roberts 2018). The network veen, ternary plots, and mantel tests were conducted in the online tool "Omicstudio" (https://www.omicstudio.cn/tool). Network veen graphs were visualized using Cytoscape 3.6.0. Redundancy analysis (RDA) and variation partitioning analysis (VPA) was performed by the online tool of Majorbio Cloud Platform (https://cloud.majorbio.com/page/tools/).

3. Results

3.1 The abundance and diversity of ARGs

A total of 151 unique ARGs and 12 MGEs were detected across all samples. The mean number of detected ARGs was 72 in the FL, which was significantly higher than the LL with a mean of 55 (Fig. 1A). In contrast, there was no difference in the detected number of ARGs across different successional stages with the mean number ranging from 65 to 69 and no significant interaction between leaf class and successional stage (Fig. 1B). The mean relative abundance of ARGs in LL was 0.14 copies per 16S rRNA gene, which was significantly higher than that of FL (0.07 copies per 16S rRNA gene) in the early stage, and there was no significant difference between LL and FL in middle and late stages (Fig.1C). The mean relative abundance of ARGs was high in the middle stage (0.08 copies per 16S rRNA gene) and low late stage in FL (0.06 copies per 16S rRNA gene), while early stage had the highest relative abundance of ARGs (0.14 copies per 16S rRNA gene) and the late stage had the lowest relative abundance of ARGs (0.05 copies per 16S rRNA gene) in LL (Fig.1D).

ARGs detected from samples were classified into eight categories based on their resistance class as described in Chen et al. (2017): Aminoglycoside, Beta-lactam, Fluoroquinolone, Multidrug, macrolide-lincosamide-streptogramin B (MLSB), Phenicol, Tetracycline, and others. Multidrug and aminoglycoside resistance genes were the most abundant ARGs detected in the leaf samples, followed by MLSB. and fluoroquinolone. The multidrug and aminoglycoside resistance genes had a higher mean proportion which was 51% and 34% across the whole sample, respectively. Further, the relative proportions of these eight categories varied across different leaves and successional stages. For instance, multidrug, phenol, and other resistance were significantly higher in litter compared to fresh leaves whilst the opposite is true for fluoroquinolone and MLSB (Fig. 1E). In relation to the successional stage, significant differences were observed for multidrug, aminoglycoside, and tetracycline, where the late stage had the highest multidrug and tetracycline but the lowest aminoglycoside (Fig. 1F).

The analysis of similarities (ANOSIM) test showed that ARG profiles significantly differed between LL and FL (Fig. 2). While the phyllosphere ARG profile in ES was significantly different from that in LS, and the ARG profiles in MS did not show any saignificant difference from in the ES and LS (Fig. 2). Further, PCoA analysis showed that the first and second principal components explained 57% of the total variation of the ARGs. The ARGs profiles changed between different leaves but were not clearly

separated between different succession stages (Fig. 2).

3.2 Shared and unique ARGs

Network veen analysis was performed to identify the shared and unique ARGs among different leaves and successional stages. There were 116 ARGs shared between FL and LL, accounting for 77% of the total ARGs. Five and 30 unique ARGs were detected in LL and FL, respectively, accounting for 3% and 20% of the total ARGs, respectively (Fig.3A). The three successional stages shared 107 ARGs (71% of total ARG numbers) with 8, 5 and 12 unique ARGs accounting for 5%, 3% and 8% of the total in ES, MS and LS respectively (Fig.3B).

3.3 Core, common and stochastic ARGs

The core ARGs included 44 ARGs that were detected in more than 80% of samples, accounting for 29% of the total ARGs and at least 72% of total ARGs abundance in each sample (Fig. 4). A total of 65 ARGs were detected in more than 50% of the samples (common ARGs) accounting for more than 43% of the total detected ARGs and at least 83% of the total ARG abundance in each sample (Fig. 4). In contrast, 86 ARGs were detected in less than half of the samples (stochastic ARGs). These stochastic ARGs account for less than 57% of the total detected ARGs and at most 17% of the total abundance of ARGs in each sample (Fig. 4). Further, these core, common and stochastic ARGs conferred resistance to all classes of antibiotics commonly administered to humans and animals, and there was a significant variation in their

detected number and relative abundance (Fig. S3).

3.4 Bacterial community

A total of 3676743 high-quality bacterial sequences were obtained from all the samples ranging from 41226 to 93678 sequences per sample. The bacterial community diversity (Shannon index) and richness (Sobs index) were significantly higher in LL than in FL (Fig. S4). Kruskal-Wallis test and analysis of community similarity showed bacterial communities significantly differed between FL and LL (Fig. S5), and microbial co-occurrence network analysis showed that LL had a high level of node number and connectivity than FL (Fig. S6). For succession stages, bacterial diversity and richness in the late stage were significantly lower than in the early and middle stages (Fig. S4). While, the phyllosphere bacterial community in ES was similar to MS, but both of them significantly differed from LS (Fig. S5). In addition, the node number and connectivity of co-occurrence network analysis decreased in the late stage than in the early and middle stages of the plant community succession (Fig. S7).

3.5 Correlation between ARGs, bacterial community, and leaf nutrient content

Mantel tests were applied to investigate core, common, and stochastic ARGs correlations between bacterial community composition and leaf nutrient content. The results suggest that leaf nutrient content and bacterial community composition tended to have a significant positive relationship with the core, common, and stochastic ARG abundance (Fig. 5A). Co-occurrence network analysis between microbial strains and ARGs showed a close interaction between them, and ARGs had a varied correlation

with different microbial taxa (Fig. 5B). *Proteobacteria* showed a stronger connection with ARGs due to its the dominant in the phyllosphere bacterial community. D*elftia, acinetobacter* and *aeromonas* were the top 3 bacterial taxa that were closely related to the ARGs in our study. RDA analysis further revealed that a total of 64% of ARG profile variation could be explained by the bacterial community and nutrient content on the first and second axes (Fig. 6A). We further conducted variation partitioning analysis (VPA) to separate the effects of bacterial community and leaf nutrients on ARG variation (Fig. 6B). These two group factors explained a total of 53.3% of the variance of ARGs. Bacterial community structure and nutrient content explained 26.8% and 8.5% of the total variation of ARGs, respectively, with 18% assigned to the interaction (Fig.6B). In addition, the ordinary least squares (OLS) linear regression demonstrates a significant positive relationship between the abundance of ARGs and MGEs (Fig. S8).

4. Discussion

4.1 The characterization of antibiotic resistome

Here we investigated the ARGs in a natural primary vegetation successional sequence using a combination of the HT-qPCR platform targetting ARGs. The results revealed that antibiotic-resistant bacteria (ARB) could widely colonize diverse plant leaves in the remote natural ecosystems that are subjected to little antropogenic disturbance, which complements previous studies that primarily focus on agricultural and urban environments. (García et al. 2020; Zainab et al. 2020). Moreover, the detected

abundances of plant phyllosphere ARGs in this natural ecosystem are at a similar level to an investigation in an urban ecosystem (Zhou et al. 2021), and covered most of the recognized antibiotics administered to human beings. These results further confirm that ARGs are spreading worldwide and have become a threat to human health in almost environmental ecosystems (Allen et al. 2010; Hernando-Amado et al. 2019).

The present study showed a high proportion (over 70%) of shared ARGs among different leaf samples, even though these leaf samples significantly differed in their phyllosphere microbial community. These results indicated that this ecosystem might have a core set of ARBs, and these key ARBs play a vital role in the resistome, as suggested by Chen et al. (2020). The presence of shared ARGs might also suggest that ARBs could move among their hosts or that selective drivers of the ARB communities focus on these shared types (Zainab et al. 2020; Zhou et al. 2021). Further, fresh leaf samples had a higher proportion of unique ARGs than leaf litter. This is likely because the fresh leaf is a fluctuant microbial habitat that is more easily affected by plant physiological state and environmental disturbance than leaf litter (Norby and Cotrufo 1998; Zheng et al. 2021), thus forming more unique ARBs under these ecological stress. Similarly, each successional stage sample also had some unique ARGs, probably due to the variation in plant species during the plant community succession (Chen et al. 2020). While interestingly, there was a relatively high proportion of shared ARGs between the early and late successional stages. The potential driving factors might be related to the intrinsic microbial community and antibiotic resistance in some plant

individuals (Chen et al. 2017). In addition, the presence of the core and stochastic ARGs in our study indicates that the plant community might have a specific selection effect on the phyllosphere ARBs. For instance, plants can influence the phyllosphere antibiotic-resistant microbial community by controlling the production of secondary metabolites, or specific microbial taxa in the phyllosphere could directly affect ARGs abundance through competition with antibiotic-producing microorganisms (Gerardin et al. 2016; Zaynab et al. 2018). However, it should be noted that there were some limitations of the shared, core, and stochastic ARGs in this study due to methodological limitations (e.g., sample coverage and use of composite samples containing a number of species), and a more systematic and comprehensive study would be needed in the future.

4.2 The distribution of antibiotic resistome and their potential drivers

Consistent with our expectations, the significantly different ARG profiles and abundances in our study indicate that, even under the same environmental conditions, host plant communities and leaf properties can strongly affect the phyllosphere antibiotic resistome. It has been suggested that plants might have the ability to attract and recruit diverse microbial populations based on their growth and developmental requirement (Theis et al. 2016; Vorholt et al. 2017). Consequently, the change in plant community across successional gradients would represent an altered preference for the microbiome, leading to a varied profile of the phyllosphere microbial community and resistome in our study. In addition, the succession of plant community could lead to an

variation of the plant speceis and their leaf functional traits, which have been found can shaped the phyllosphere microbial community composition (Kembel et al. 2014; Li et al. 2023). As a consequence, our results found that the phyllosphere bacterial community composition and diversity significantly varied during the succession of plant community. Further, our results showed that the abundance of ARGs in the phyllosphere significantly decreased during the plant community succession process. This is likely caused by the variation of the phyllosphere microbial community composition, because the reduced bacterial diversity during plant community succession in our samples might influence ARB by regulating competition or metabolite presence (Pärnänen et al. 2019; Zhou et al. 2021), leading to a relatively lower ARG abundance in the late-successional stage in our study. Moreover, the varied microbial biomass and disturbance from visitors in the early than in the late s successional stage site might also contribute to the reduced the ARGs abundance during the plant community succession (Jiang et al. 2018a; Urra et al. 2019; Zhang et al. 2022a). Further, the interaction between different microbial groups is also one of the main drivers of the microbial population since microorganisms can cooperate or exclude each other (Falony et al. 2016; Zhu et al. 2020). Correspondingly, our results showed a close connection between microbial strains and ARGs. Thus, the decreased microbial community complexity during the plant community succession process in our study might also drive the changes in the ARG profiles and the lower ARG abundance in the late successional stage. In addition to the direct effect, leaf nutrient content could also shape the phyllosphere bacterial community, as suggested by Kembel et al. (2014), and

thus indirectly regulate the ARB profiles. This was confirmed by the results that bacterial community composition and leaf nutrient content together explained over half of the variation of ARG profiles in our study.

The local dispersal of the microorganisms is one of the important determinants of microbial community composition and abundance (Meyer et al. 2022).Our results showed a higher abundance of ARGs in leaf litter than in fresh leaf. Generally, soil contains a relatively higher microbial biomass and abundance than in leaves, due to the soil has a more stable microenvironment and rich nutrients (Allen et al. 2010; McEachran et al. 2015; Zhang et al. 2022b). Although the nutrient content in fresh leaf is higher than in leaf litter, the closer physical contact and higher interactions between soil and leaf litter resulting in a relatively higher microbial biomass and ARG abundance in LL than in FL in our study. In addition, the relative lower detected number of ARGs in LL than in FL probably related to the community stability and the dominant species of the micorbial community (Chen et al. 2020; Theis et al. 2016). Correspondingly, our results found that LL a higher bacterial community stability and a higher proportion of the dominant species than in FL. Further, the MGEs abundance was significantly correlated with the ARGs abundance in our study, indicating that the MGEs may also play a role in determining the ARG profile through horizontal gene transfer of antibiotic resistance. This is supported by previous studies that the conjugative plasmid transfer facilitates ARG dissemination in different environments (Chen et al. 2017; Dharmarha et al. 2019). However, it should also be noted that the

leaf surface is exposed to rapid environmental fluctuation linked to stochastic events, and those uncertain factors could also drive the phyllosphere antibiotic-resistance bacterial community assembly and the ARG profiles (Yan et al. 2021; Zhu et al. 2021b).

5. Conclusion

Compared with highly disturbed urban and agricultural ecosystems, the phyllosphere in the natural ecosystem with less antropogenic disturbance also harbors a broad spectrum of ARGs. Our results further demonstrate a high proportion of the detected shared ARGs among different leaf samples, with a core set of ARGs accounting for more than 72% of the total abundance in this natural system. The succession of plant communities leads to variations of ARG profile and lower ARG abundance in the late-successional stage. In addition, the change in leaf properties and surrounding habitat also leads to variations in the phyllosphere ARG abundance and profiles. Furthermore, the phyllosphere ARG profile and its abundance are linked to both the phyllosphere bacterial community and leaf nutrient content. This study improves our knowledge regarding plant resistome and provides novel insights into the composition and dynamics of ARGs in natural terrestrial ecosystems.

Declaration of competing interest

The authors declare no conflict of interest.

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Table 1. Fresh leaf and leaf litter nutrient content during the plant community succession process.

		Carbon (mg g^{-1})	Nitrogen (mg g^{-1})	Phosphorus (mg g^{-1})	Potassium (mg g^{-1})	Sulphur (mg g^{-1})
Earely Stage	Fresh Leaf	444.2±4.0	24.9±1.9	2.0±0.1	14.2±1.1	2.1±0.1
	Leaf Litter	266.2±29	18.0±1.8	0.9±0.1	2.3±0.2	1.8±0.2
Middle Stage	Fresh Leaf	448.0±4.6	18.6±0.8	1.5±0.1	15.2±2.1	2.0±0.1
	Leaf Litter	323.3±17	18.6±0.9	0.9±0.1	1.9±0.2	1.9±0.1
Late Stage	Fresh Leaf	465.2±9	12.2±0.5	1.0±0.1	5.7±0.5	1.2±0.1
	Leaf Litter	276.3±41	15.2±2.0	0.7±0.1	1.9±0.3	1.6±0.2
ANOVA *P* value	Successional stage	**>** 0.05	**<** 0.001	**<** 0.001	**<** 0.001	**<** 0.001
	Leaf fall	**<** 0.001	**>** 0.05	**<** 0.001	**<** 0.001	**>** 0.05

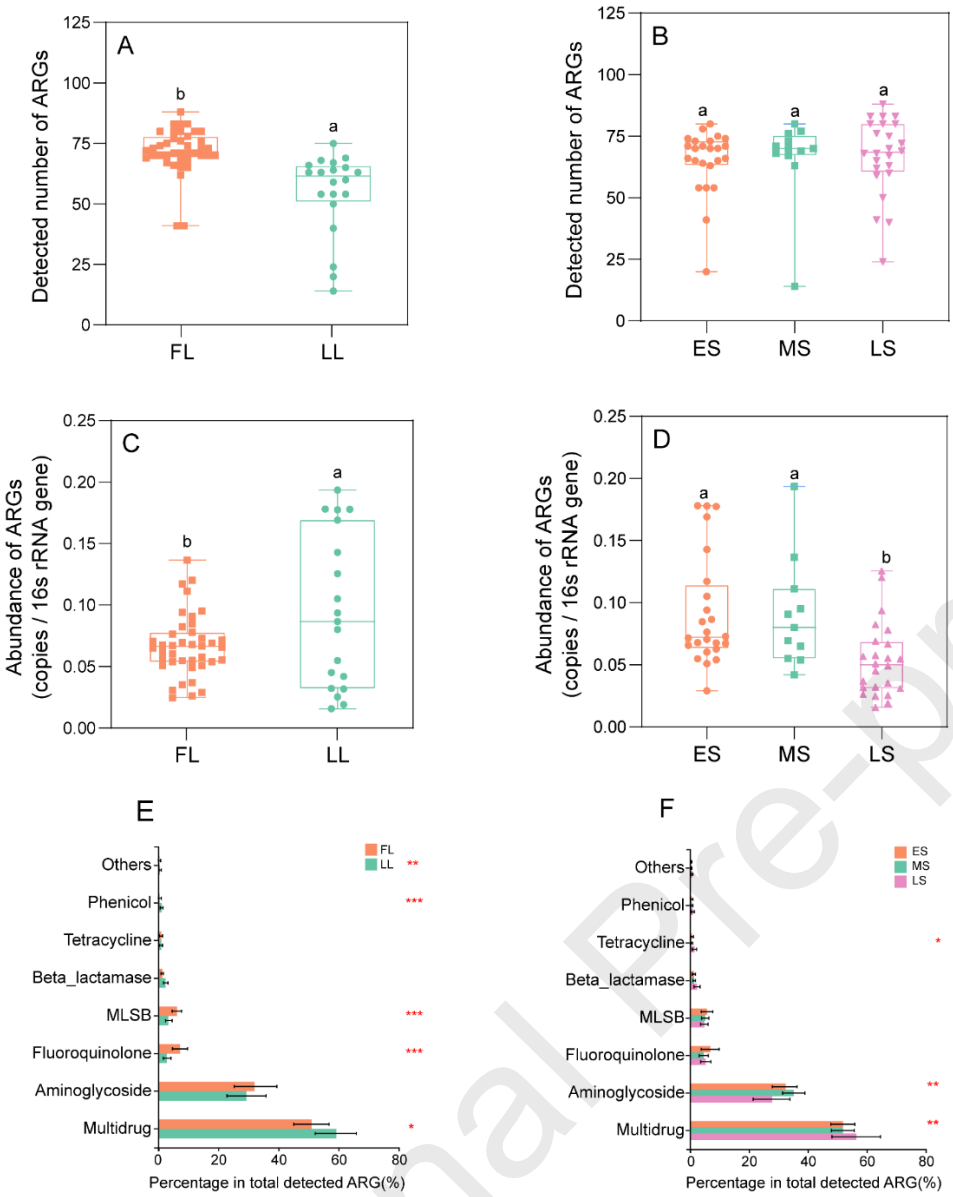

Fig.1. Comparison of the profile of antibiotic resistance genes (ARGs) by the box plot with the median. (A) and (B) show the analysis of ARG richness with ANOVA results displayed; (C) and (D) show the detected relative abundance of ARGs in different leaves and succession stages. Different letters indicate significant differences at $P < 0.05$ level (ANOVA) following post-hoc Tukey tests. (E) and (F) were the Kruskal-Wallis test of ARGs that were classified based on their drug class. Leaf classes: FL – fresh leaf, LL – leaf litter. Successional stage: ES – early stage, MS – middle stage and LS – late stage.

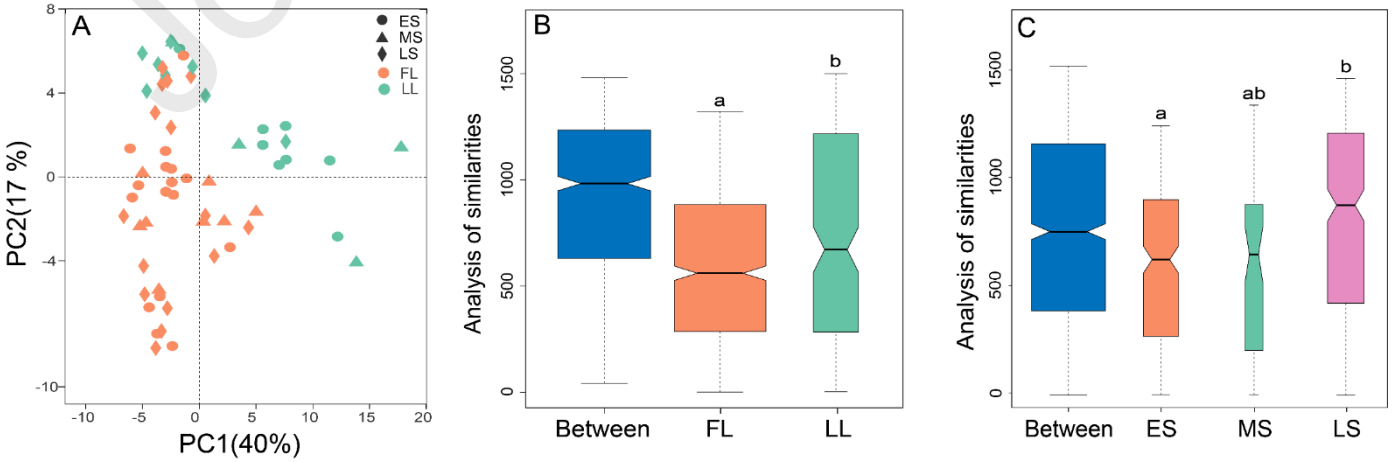

Fig.2. (A) Principal Coordinate Analysis (PCoA) of the antibiotic resistance genes, (B) and (C) showed the analysis of similarities (ANOSIM) of the ARG profile in different succession stages and leaf classes. Leaf classes: FL – fresh leaf, LL – leaf litter. Successional stage: ES – early stage, MS middle stage and LS – late stage. Different letters indicate significant differences at $P < 0.05$ level.

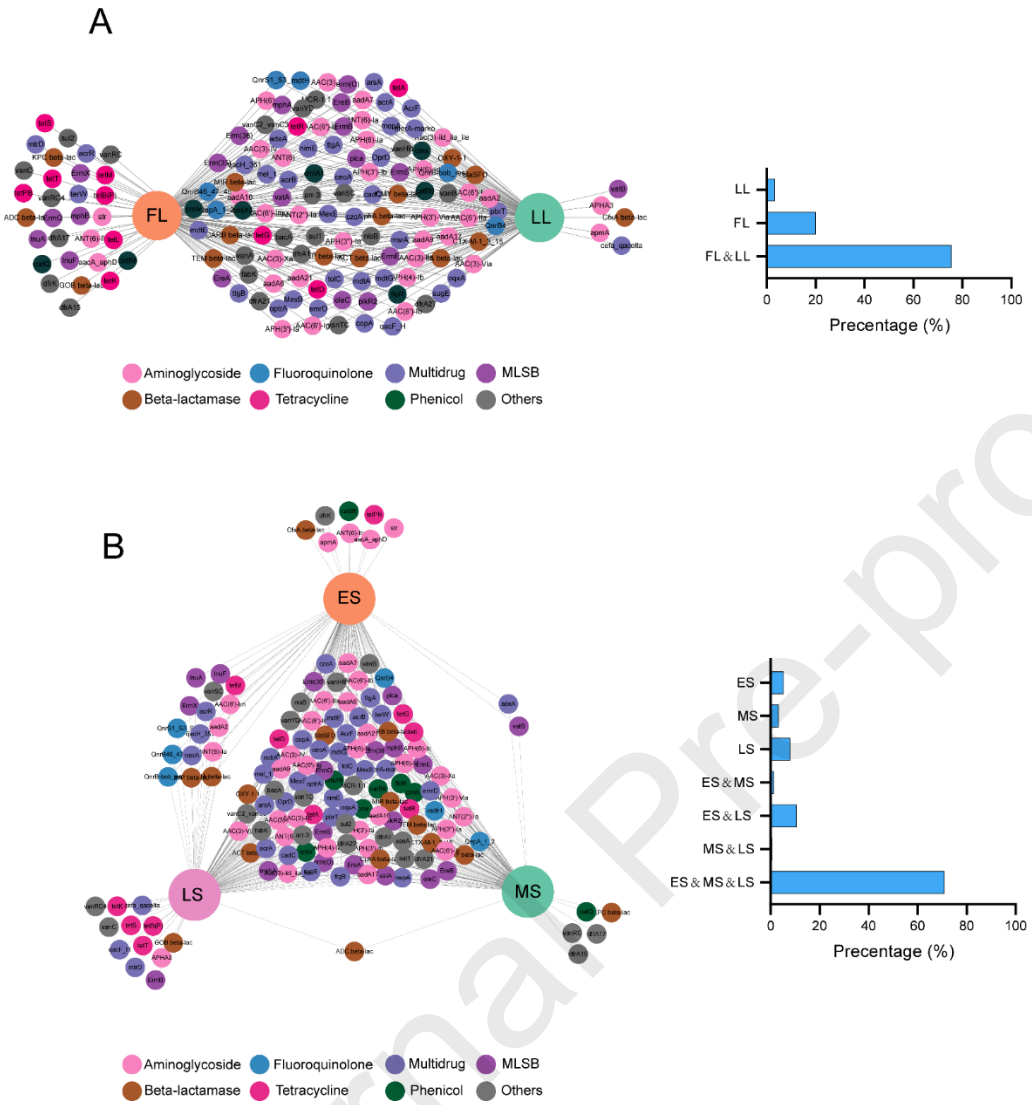

Fig.3. Network veen analysis reveals the shared and unique antibiotic resistance genes (ARGs) profile in different leaf calsses (A) and succession stages (B). Leaf classes: FL – fresh leaf, LL –leaf litter. Successional stage: ES – early stage, MS – middle stage and LS – late stage. ARGs were classified based on the drug class.

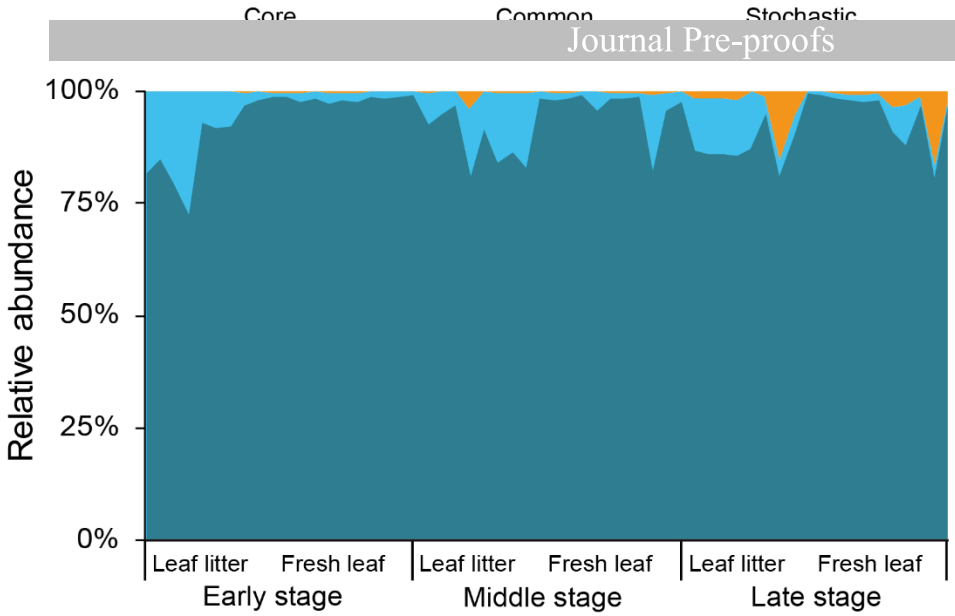

Fig.4 The distribution of the core, common and stochastic antibiotic resistance genes in different leaves and succession stages. The area plots display the percentage changes.

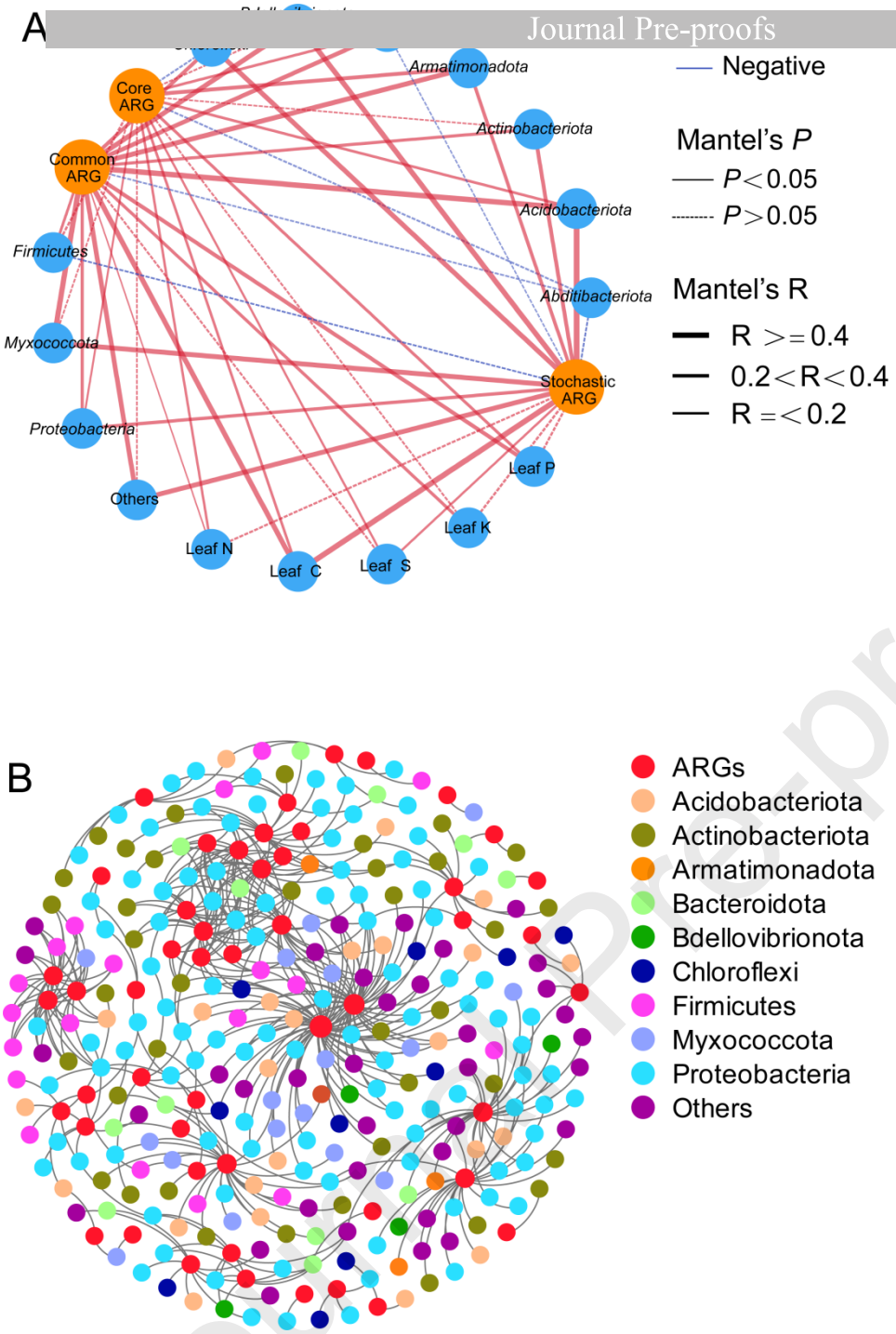

Fig.5. (A) Relationship between antibiotic resistance genes (ARGs), bacterial community composition, and leaf nutrient contents. ARGs were displayed in the core, common and stochastic groups. (B) Co-occurrence networks analysis between antibiotic resistance genes (ARGs) and bacterial communities. Edges represent significant co-occurrence relationships (Spearman's $\rho > 0.8$ and $P < 0.05$).

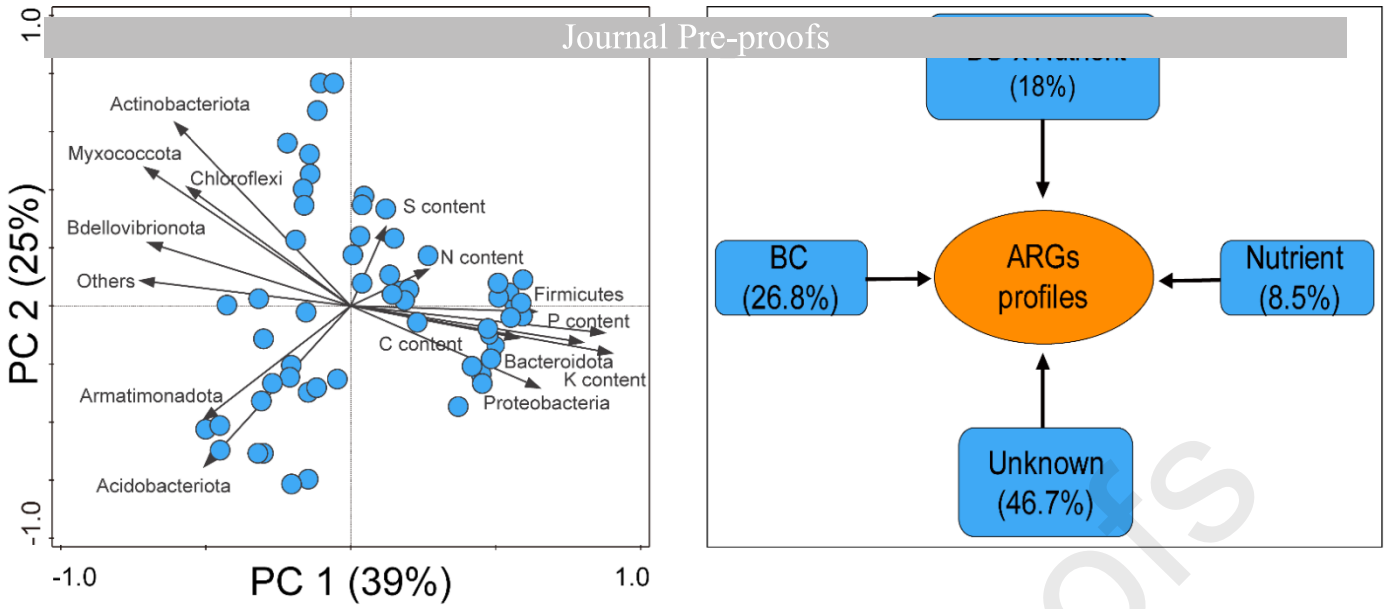

Fig.6. Role of the bacterial community (BC) and nutrient content in shaping the antibiotic resistance genes (ARGs). (A) Redundancy analysis (RDA) of the relationship between bacterial community, nutrient content, and ARGs. (B) Variation partitioning analysis (VPA) differentiates bacterial community and nutrient content effects on the ARGs profile.

Author contributions:

JL, TW, R-YC, X-RY and Y-GZ designed the study. JL, TW, R-YC, and JZ managed the field trial stations and collected samples. JL, M-KJ and JZ conducted the laboratory analyses and performed the data processes. JL, T-JD, X-RY and Y-GZ wrote the manuscript. All authors read and approved the final manuscript.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: