Environmental factors influencing the composition of phyllosphere bacterial communities in bamboo: A staple food source of giant pandas

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Abstract

Background

The giant panda has developed a series of evolutionary strategies to adapt to a bamboo diet. The abundance and diversity of the phyllosphere microbiome change dramatically depending on the season, host species, location etc., which may, in turn, affect the growth and health of host plants. However, few studies have investigated the factors that influence phyllosphere bacteria in bamboo, a staple food source of the giant panda.

Methods

Amplicon sequencing of the 16S rRNA gene of rRNA genomic loci was used to explore the abundance and diversity of phyllosphere bacteria in three bamboo species (*Arundinaria spanostachya*, *Yushania lineolate* and *Fargesia ferax*) over different seasons (spring *vs.* autumn), elevation, distance from water, etc. in Liziping National Nature Reserve (Liziping NR), China.

Results

The results show that a total of 2,562 operational taxonomic units (OTUs) were obtained from all 101 samples, which belonged to 24 phyla and 608 genera. *Proteobacteria* was the dominant phyla, followed by *Acidobacteria* and *Actinobacteria*. The Sobs index and Shannon index of *F. ferax* phyllosphere bacteria were greater than that of the other two bamboo species in both seasons. The Sobs index and Shannon index of phyllosphere bacteria in all three bamboo species in autumn were significantly higher than in spring. Season was a stronger driver of community structure of phyllosphere bacteria than host bamboo species based on the (un)weighted UniFrac distance matrix. Many bacteria phyla were negatively correlated with elevation and distance from water, but positively related to mean height of bamboo and mean base diameter of bamboo. Function prediction of PICRUSt revealed the relative abundance of transporters function was highest in all three bamboo species, followed by ABC transporters. There were nine relative abundance pathways with significant differences in the 3-level KEGG pathway. The genes related to membrane transport, signal transduction and porphyrin transport in phyllosphere bacteria of *F. ferax* were significantly lower than in the other two species.

Conclusions

The composition, diversity and community structure of phyllosphere bacteria in bamboo, a staple food source of giant pandas, were primarily affected by the season, species, altitude, tree layer and shrub layer. The better the growth of bamboo forests, the richer the bacterial phyla in the bamboo phyllosphere. Our study presented a deeper understanding of factors influencing the bacterial community in the bamboo

phyllosphere. These findings could provide a reference for the restoration and management of giant panda habitat and food resources in this area, especially for those small isolated populations of giant pandas in Xiaoxiangling mountains.

Background

Large numbers of different kinds of microorganisms within the phyllosphere constitute a stable and complex micro-ecosystem (Mei et al. 1991; Li et al. 1998; Shi et al. 2007; Vorholt et al. 2012). Different hosts have different microbial communities (Yadav et al. 2008; Leveau et al. 2015) and may include bacteria, archaea, fungi and protists (Newton et al., 2010). Bacteria is the most abundant microorganism in the phyllosphere (Lindow et al., 2003) and Proteobacteria are the dominant phylum (Delmotte et al. 2009).

Numerous environmental factors determine phyllosphere community composition, such as plant species, temperature, humidity, nutrient availability (on the plant surface), sun/UV exposure levels, and even the underlying soil geochemistry (Lindow et al. 2003; Leveau et al. 2015). Ecological factors and plant attributes e.g., wood density, leaf mass per unit area, and leaf nitrogen and phosphorus concentration, may also affect the microbial community structure in the phyllosphere (Steven et al. 2014).

The phyllosphere microbial community represents a wide range of primitive symbiotic relationships (Fedorov et al. 2011; Partida et al. 2011). Phyllosphere microbiota can affect plant yield (e.g., via nutrients or hormones) (Gourion et al. 2006; Reed et al. 2010) and plant health in a variety of ways (Balint et al. 2010; Innerebner et al. 2011). For example, *Beauveria* fungus on rice leaf can protect rice enzyme activity and is an environmentally friendly microbe (Du et al. 2014). Some phyllosphere microbes are beneficial to plants via nitrogen fixation (Leveau et al. 2015), growth promotion (Bringel et al. 2015), acting as barriers against pathogen invasion, and decomposition of residual pesticides (Venkatachalam et al. 2016).

Bamboo is a perennial evergreen plant belonging to the Gramineae family and Bambusoideae subfamily and is an important forest resource all over the world. It has the characteristics of wide-distributing, fast-growing and high-yield, and strong regeneration ability. Therefore, it has considerable economic, ecological and social benefits (Shanmughavel et al. 1997; Anu et al. 2008; Chang et al. 2015; Zhang and Xue, 2018). Bamboo leaves are an important habitat for many microorganisms, with microbial abundance and diversity acting as an indicator of forest health. However, few studies have investigated the environmental factors that influence phyllosphere bacterial composition in bamboo. Using the traditional cultivation method, Zhang and colleagues (2014) found that the composition of the phyllosphere microbial community differed between bamboo species and seasons. The species composition and frequency of leaf endophytic bacteria and fungi have also been found to differ between bamboo species (Helander et al. 2013), while endophytic fungi from fish-scale bamboo (*Phyllostachys heteroclada*) differs between the branch and leaf tissue (Zhou et al. 2017). Significant differences in bacterial richness and diversity were also observed between different bamboo species using high

throughput amplicon sequencing (Jin et al. 2020). However, there is a lack of research on what factors influence the composition and diversity in the bamboo phyllosphere microbiome.

The giant panda (*Ailuropoda melanoleuc*) belongs to a carnivorous clade (Wei et al. 1999), yet has an exclusively herbivorous diet and specialises in the consumption of bamboo leaves throughout the year (Hu et al. 1985; Zhao et al. 2013). In the long evolutionary process, the giant panda developed a series of foraging strategies to adapt to a bamboo diet, such as seasonal vertical migration, selection of habitat and feeding point (Hong et al. 2015, 2016). However, in the different seasons of different mountain systems, the diet of giant pandas does vary. For example, in the Qinling mountains, giant pandas mainly feed on bamboo leaves during the non-bamboo shoot seasons (Pan, 2001; Wu et al. 2017), while bamboo leaves of the Xiaoxiangling mountains account for more than half of the panda faeces in summer and autumn (Wei et al. 1999). Although bamboo is an important food source, its leaves also contribute to intestinal diseases of giant pandas in captivity. Previous research shows that *Escherichia coli* and *Klebsiella pneumoniae* could cause diarrhoea and septicaemia for giant pandas (Zhang et al. 1997; Xiong et al. 1999). However, there are few studies into the phyllosphere microbiome in the bamboos pecies foraged by wild giant pandas.

In this study, we investigate the phyllosphere bacterial community of bamboo species frequently used as a food source by giant pandas, using Next Generation Sequencing Technology (NGS) in Liziping National Nature Reserve (Liziping NR), Sichuan, China. The specific goals include: (i) comparing differences in microbial composition and diversity between three bamboo species and two seasons; (ii) exploring the ecological factors that influence the phyllosphere bacteria community changes; and (iii) predicting the functional differences of phyllosphere bacterial communities in different bamboo species.

Materials And Methods

Study area

Liziping NR is located in Shimian County, Sichuan Province, China, and is in the middle and upper reaches of the Dadu River, on the southwestern edge of the Sichuan Basin and southeast of Gongga Mountain (E102°10'33"-E102°29'07", N28°51'02" -N29°08'42") (Hong et al., 2015). The reserve covers an area of 47940 km^2 and ranges from 1330 m to 4550 m above sea level, with uneven ridges and narrow valleys. The annual average temperature and rainfall are 11.7–14.4°C and 800–1250 mm respectively (Xie et al., 2020). As the altitude increases, the vegetation in the reserve transitions from evergreen broad-leaved forest to deciduous broad-leaved forest, then coniferous and broad-leaved mixed forest, coniferous forest to alpine thickets and alpine rocky beech. According to the Fourth National Survey of giant pandas, there were 22 giant pandas in the reserve in 2015 (Sichuan Forestry Bureau. 2015). To strengthen the small isolated populations of giant pandas, the Chinese government initiated the Captive Giant Panda Release Program (CGPRP). To date, one giant panda rescued from the wild and nine giant pandas from captivity have been released into this nature reserve (Hong et al., 2012). There are seven bamboo species (three genera) consumed by the giant panda within the Xiaoxiangling Mountains. The

species with the largest distribution is *Arundinaria spanostachya*, which accounts for 38.08% of the total area of giant panda feeding bamboos in these mountains. Next is *Yushania lineolate* and *Fargesia ferax*, which account for 28.02% and 12.48% of the giant panda's feeding bamboo respectively (Sichuan Forestry Bureau. 2015). *A. spanostachya* mainly grows above 2500 m a.s.l, while *Y. lineolate* and *F. ferax* occur below 2800 m a.s.l. Giant pandas in this nature reserve prefer to eat *A. spanostachya* throughout the whole year, some *Y. lineolate* in winter, and occasionally *F. ferax* (Hong et al., 2015, 2016; Xie et al., 2020).

Experimental design

We conducted surveys and sampling during May (Spring) and October (Autumn) in 2020. First, we set up four transects in *A. spanostachya*, *Y. lineolate* and *F. ferax* bamboo forests respectively. The transects were set from low altitude to high altitude, and the distance between them was no less than 200 m. Secondly, we set up 3–5 survey plots (20×20 m^2) in each transect, with the altitude distance of adjacent survey plots on the same transect no less than 50 m. We then recorded and measured bamboo species, latitude and longitude, altitude, and other related variables in the tree and shrub layer (Table S1). Moreover, one bamboo plot (1×1 m^2) was set up in the centre of each survey plot, and another two bamboo plots (1×1 m^2) were set up east and south, 5 m from the centre point of each survey plot. Finally, the related variables within the bamboo layer were also measured and recorded (Table S1).

Sample collection and DNA extraction

In each survey plot, one mixed bamboo leaf sample (not less than 200g) was collected with sterile gloves, immediately transported to the laboratory (less than 2 hours) and stored at -20°C for further processing within 48 h. Each 200g sample was aseptically transferred into a Ziplock bag (24 cm × 35 cm) containing 200 ml sterile precooled TE-buffer (10 mM Tris, 1 mM EDTA, pH 7.5) supplemented with 0.05% Tween-80 (Hong et al. 2017). Leaf surfaces were washed to collect the microbial population by 5 min of shaking, vortexing and sonication of each sample in the TE-buffer, with the Ziplock bag kept in ice water (~ 4°C) for each processing step (Hong et al. 2017). The cell suspension was separated from the leaf material by filtration through three-layer sterile nylon mesh. Sonication was performed at a frequency of 40 kHz in an ultrasonic cleaning bath (Shanghai Kudos Instrument Co. Shanghai, China) to dislodge the microbes from the leaf surface. Following filtration, cell suspensions were placed in four 50 ml tubes/sample, and cells were pelleted using centrifugation at 2000×g for 15 min at 4°C. Cell pellets from multiple tubes were pooled into 2.0-ml reaction tubes and washed twice with TE-buffer with Tween-80. Cell pellets were immediately frozen at − 80°C until DNA extraction.

DNA extraction was performed using the E.Z.N.A.™ Soil DNA Kit (Omega, Norcross, GA) as described with slight modifications. Frozen cell pellets were resuspended in 1 ml of kit-supplied SLX Mlus buffer with 500 mg of glass beads, and cell lysis was performed at 65 Hz for 90 s. The cell debris suspension was immediately processed following the instructions in the kit manual. Finally, total DNA was obtained from the column by two sequential elutions with 50 µl elution buffer.

16S rRNA gene V3-V4 amplification, quantification and sequencing

Sequencing of the V3-V4 hypervariable region of the 16S rRNA gene was performed for bacterial identification (Caporaso et al. 2011; Lundberg et al. 2013). Thirty-five cycles of polymerase chain reaction (PCR) amplification of the target marker genes were performed. Error-correcting 12-bp barcoded primers specific to each sample were used to permit multiplexing of samples. Polymerase chain reaction products from all samples were quantified using the PicoGreen dsDNA assay and pooled together in equimolar concentrations. Each library was submitted to Mega Biotech on the Illumina MiSeq PE300 platform. The raw reads were deposited in the NCBI Sequence Read Archive (SRA) database with accession number PRJNA718425.

Sequence data analysis

The raw 16S rRNA gene sequencing reads were demultiplexed, quality-filtered by fastp version 0.20.0 (Chen et al.,2018) and merged by FLASH version 1.2.7 (Magoč et al.,2011) with the following criteria: (i) the 300 bp reads were truncated at any site receiving an average quality score of < 20 over a 50 bp sliding window, and the truncated reads shorter than 50 bp were discarded. Reads containing ambiguous characters were also discarded; (ii) only overlapping sequences longer than 10 bp were assembled according to their overlapped sequence. The maximum mismatch ratio of the overlap region was 0.2. Reads that could not be assembled were discarded; (iii) samples were distinguished according to the barcode and primers. The sequence direction was adjusted using exact barcode matching with two nucleotide mismatch in primer matching.

Operational taxonomic units (OTUs) with 97% similarity cut-off (Edgar,2013; Stackebrandt et al.,1994) were clustered using UPARSE version 7.1 and chimeric sequences were identified and removed. The taxonomy of each OTU representative sequence was analysed by RDP Classifier version 2.2 (Wang et al., 2007) against the 16S rRNA database (e.g., Silva v138) using a confidence threshold of 0.7. Non-target sequences, including mitochondrial and chloroplast sequences, were also removed by QIIME from the final OTU data set. To better convey the biological information in these samples, the average relative abundance of the bacterial community was visualized by bar at the level of phylum and genus.

Statistical analysis

First, Kruskal-Wallis H tests and Wilcoxon rank-sum tests were used to evaluate the differences between dominant bacteria abundance at both phylum and genus levels, for each combination of bamboo species and season. We used mothur software (version v.1.30.1) to calculate the Sobs and Shannon indices for each sample, to estimate the species abundance and diversity of phyllosphere bacteria between the different bamboo species and seasons and used a Student's t-test to identify any significant differences. We then used PCoA based on the weighted UniFrac and unweighted UniFrac method distance matrix to evaluate the differences in the microbial community structure between the bamboo species and seasons. We also used the permutational MANOVA (PERMANOVA) to analyse the effect of different bamboo

species and seasons on the phyllosphere bacterial community, based on Bray-Curtis distance matrices with a substitution test to analyse the statistical significance of the division.

Second, Spearman's correlation heatmap analysis was performed to examine the relationship between the relative abundance of bacterial taxa and the environmental factors described in Table S1. Linear regression was used to evaluate the relationship between environmental factors and the results of either Alpha (Sobs and Shannon indices) or Beta diversity analysis (Bray-Curtis distance). The Mantel test was used to test the correlation between the UniFrac distance matrix and the environmental variable distance matrix.

Finally, the PICRUSt prediction was used to predict the functional composition of all phyllosphere bacteria in the different bamboo species. The greengene id corresponding to each OTU, the COG and KEGG functions of the OTU were annoted to obtain the function level of COG and KEGG, and the abundance information for each function in different samples.

Results

Samples, sequences and OTUs between different seasons and bamboo species

Up to 19 samples of each bamboo species were collected in each study season (Table 1). After removing the mitochondrial and chloroplast sequences, a total of 1,233,917 effective target 16S rRNA reads were obtained. The average sequence of each sample was 12,217 ± 3,037. Clustered by 97% similarity, all samples had a total of 2,564 OTUs. 1378 OTUs were shared between the three bamboo species, and the bacterial OTUs of each in autumn were significantly higher than in spring (Fig. 1 and S1). *Fargesia ferax* had a higher number of OTUs than the other two species in both seasons (Fig. 1a).

Table 1
Statistics table of each sample and sequence (AS: *A. spanostachya*; YL: *Y. lineolate*; FF: *F. ferax*) (Mean ± SD).

Season	**Spring**			**Autumn**		
Banboo specie	AS	YL	FF	AS	YL	FF
Samples	17	17	16	19	16	16
Total number of sequences	41,426 ± 5,012	45,313 ± 5,317	43,209 ± 4,450	53,715 ± 4,620	51,920 ± 2,923	52,921 ± 6,892
Total number of effective sequences	23,149 ± 2,291	24,619 ± 3,70125	20,013 ± 3,572	24,799 ± 1,453	25,286 ± 797	24,098 ± 2,170

The influence of season and bamboo species on phyllosphere bacterial composition

After the clustered OTU representative sequence was annotated for species, all bacterial OTUs belonged to 24 phyla and 614 genera. The most dominant phylum was Proteobacteria, comprising ~ 70% of the bacterial diversity observed across all samples (Fig. 2a). The remaining bacteria were distributed among the phyla Acidobacteria, Bacteroidota, Actinobacteria, Planctomycetota, Myxococcota and others. In spring, the relative abundance of phyla Bacteroidota, Actinobacteriota and Myxococcota in the *F. ferax* phyllosphere were significantly higher than that of *A. spanostachya* and *Y. lineolate*. However, the relative abundance of phylum Acidobacteriota in the *F. ferax* phyllosphere was significantly lower than that of *A. spanostachya* and *Y. lineolate* (Fig. 2a; Table S2). In autumn, the relative abundance of phyla Actinobacteriota and Myxococcota in the *F. ferax* phyllosphere were significantly higher than that of *A. spanostachya* and *Y. lineolate*. The relative abundance of phyla Bacteroidota in *Y. lineolate* and Planctomycetota in *A. spanostachya* phyllospheres were significantly lower than that of the other two bamboo species (Fig. 2a; Table S2). Not difference was found in the relative abundance of dominant phyla in the *F. ferax* phyllosphere between seasons, except Planctomycetota (Table S2). The relative abundance of phylum Acidobacteriota in the *A. spanostachya* and *Y. lineolate* phyllospheres were lower in autumn than spring, and the relative abundance of phyla Bacteroidota, Actinobacteriota and Myxococcota all increased from spring to autumn (Table S2).

The relative abundances of genera 1174-901-12, *Acidiphilium*, *Sphingomonas*, unclassified_f__Acetobacteraceae, *Terriglobus*, *Granulicella*, *Pseudomonas* and *Hymenobacter* revealed them to be the dominant bacteria in the phyllosphere of all three bamboo species, and the differences among the top eight total abundances of genera were calculated. In spring, the relative abundance of 1174-901-12, *Acidiphilium* and *Terriglobus* were highest in the *Y. lineolate* phyllosphere. The relative abundance of *Hymenobacter* and *Sphingomonas* were highest in the *F. ferax* phyllosphere, but the opposite pattern was observed for *Granulicella* (Fig. 2b; Table S2). In autumn, the lowest relative abundance of *Acidiphilium* and *Granulicella* existed in the *F. ferax* phyllosphere, and that of *Hymenobacter* and *Sphingomonas* existed in the *Y. lineolate* phyllosphere (Fig. 2b; Table S2). For all three bamboo species, similar relative abundance patterns were found for the seven most dominant bacteria genus in each phyllosphere. The relative abundance of 1174-901-12, *Acidiphilium*, *Hymenobacter*, *Granulicella* and *Terriglobus* decreased from spring to autumn but increased for *Sphingomonas* and *Pseudomonas* (Fig. 2b; Table S2).

The influence of season and bamboo species on phyllosphere bacterial diversity. The Sobs and Shannon indices for the bacterial community in the *F. ferax* phyllosphere were higher than that of *A. spanostachya* and *Y. lineolate* in both seasons (Fig. 3; Table S3). However, in spring the Sobs and Shannon indices in *Y. lineolate* was lower than that of *F. ferax* and *A. spanostachya*. The phyllosphere bacterial community of *A. spanostachya* showed the lowest Sobs and Shannon indices in autumn (Fig. 3; Table S3). These indices were also found to increase significantly from spring to autumn in all bamboo species (Fig. 3).

The results of the PCoA revealed that samples were clustered by species and season. Samples of *A. spanostachya* and *Y. lineolate* were also clustered together away from that of *F. ferax* in the same season (Fig. 4). Principle Coordinate Analyses based on unweighted UniFrac differentiated better the samples

based on weighted UniFrac (Fig. 4). The PERMANOVA revealed significant differences in phyllosphere bacterial community based on Bray-Curtis distance matrixes between the three bamboo species in spring and autumn (Table S4).

The influence of ecological factors on bamboo phyllosphere bacterial community

The results of Mantel analysis revealed that elevation had the greatest impact on the phyllosphere bacterial community ($R = 0.313$, $P = 0.001$). And elevation; distance from water; trees height; trees diameter at breast height; shrubs coverage; shrubs numbers; mean height of bamboo and mean base diameter of bamboo also had significant impacts on the bacterial community (Table S5).

Spearman's correlation heatmap analysis revealed that almost all bacterial phyla abundance were positively correlated with the base diameter of bamboo except Proteobacteria and Actinobacteria, and almost all of the bacterial phyla abundance were negatively correlated with elevation except Proteobacteria, Acidobacteria and WPS-2 (Fig. 5a). Moreover, distance from water was negatively correlated with abundance of Abditibacteriota, Actinobacteria, Bacteroidota and Deinococcota, but positively correlated with Acidobacteria and WPS-2. Abundance of Bdellovibrionota was positively correlated with tree height, trees diameter at breast height, shrub coverage and the number of shrubs. Mean height of bamboo was positively correlated with Chloroflexi, Fusobacteriota, Myxococcota, SAR324_cladeMarine_group_B, unclassified_k_norank_d_Bacteria and Verrucomicrobiota, but negatively correlated with WPS-2. Trees diameter at breast height was negatively correlated with unclassified_k_norank_d_Bacteria and positively correlated with WPS-2. At the genus level, 1174-901-12 and *Acidiphilium* were both negatively correlated with tree height, trees diameter at breast height, shrub coverage and shrubs number (Fig. 5b). A positive correlation was observed between *Sphingomonas* and shrubs number, shrubs number, tree height and mean base diameter of bamboo. G*ranulicella* showed significant correlations with elevation and water source distance, but negative correlations with mean base diameter of bamboo (Fig. 5b).

Further linear regression found that elevation, bamboo deaths, mean base diameter of bamboo, bamboo coverage and tree height had a significant impact on the Sobs and Shannon indices (Table S6). Elevation had the highest explanation for the Sobs index, the highest explanation ecological factor for the Shannon index, and Bray-Curtis distance was death bamboo number (Table S6). Elevation, death bamboo number, mean base diameter of bamboo, bamboo coverage and tree height, shrub coverage and shrubs number all had significant relationships with bacterial community structure in bamboo phyllosphere based on Bray-Curtis distance matrixes (Table S6).

PICRUSt gene function estimation

All three bamboo phyllosphere bacterial communities had similar COG function classification patterns as generated by PICRUSt in both spring and autumn (Fig. S2). There were higher relative abundance sequences related to cell wall/membrane/envelope biogenesis, amino acid transport and metabolism.

Interestingly, the relative abundance of transporter function (4.17%) was the highest in all three bamboo species, followed by ABC transporters (2.55%), DNA repair and recombination proteins (2.37%) and two-component systems (2.23%). Prediction software PICRUSt enriched 22 categorizable dominant pathways (relative abundance > 1%) in the level 3 KEGG pathway (Fig. 6; Table S7). Among them, nine pathways had significant differences between bamboo species ($P < 0.05$). It is worth noting that there were significant differences in the relative abundance of the bacterial secretion system, the secretion system and the two-component system. Oxidative phosphorylation in energy metabolism, porphyrin and chlorophyll metabolism were also different, and the gene relative abundance for these functions for *F. ferax* was significantly lower than that of the other two species (Fig. 6; Table S7).

Discussions

This study revealed that the bacterial OTU richness of the three bamboo species in autumn was significantly higher than that in spring. The Phyllosphere of *F. ferax* had a greater diversity of bacterial OTUs than that of *A. spanostachya* and *Y. lineolate*. The dominant phyllosphere bacteria of the three bamboo species are *Proteobacteria*, *Acidobacteria*, *Bacteroides* and *Actinomycetes*. In a warm and humid climate, the diversity and richness of phyllosphere bacteria in the three bamboo species in spring were significantly higher than in autumn. The overall importance of seasonality to the structure and composition of the phyllosphere microbial community has been confirmed by many studies (Thompson et al., 1993; Copeland et al., 2015). *Proteobacteria*, *Acidobacteria*, *Bacteroides* and *Actinomycetes* are often detected in a variety of forests, indicating that these organisms have a wide ecological range and an ability to adapt to many environments (Isabelle et al., 2016; Feng et al., 2019). In this study, the relative abundance of the *Proteobacteria* in the three bamboo species were all above 60%, and the differences between the bamboo species were not significant, indicating that *Proteobacteria* played a dominant role in the phyllosphere microbial community. Its changing in turn may impact health of the staple food bamboo foraged by giant panda around Xiaoxiangling mountains. In spring, the relative abundance (15.29%) of *Acidobacteria* in *F. ferax* was significantly lower than that of *A. spanostachya* (21.16%) and *Y. lineolate* (25.67%). *Actinomycetes* are gram-positive bacteria that can decompose cellulose and lignin (Taibi et al., 2012).

The bacterial diversity and abundance of all three bamboo species in autumn were significantly higher than that in spring, which was similar to the result of Zheng and colleagues (2011), who found that the number of phyllosphere microbial community of *Pinus tabulaeformis* varied significantly between different seasons, with the largest diversity and abundance in autumn, followed by summer and the least in spring. The higher temperature and humidity in summer and autumn contributed to the higher diversity and richness than that in spring, while the higher altitude and longer low temperature in winter lead to the lower diversity and abundance (Isabelle et al, 2016). Airborne microorganisms can settle directly onto the leaf layer and their diversity and density may vary with time (daily and seasonal patterns)and other environmental events (Liu et al., 2020). In addition, agricultural practices such as harvesting and planting also have an impact on the movement of airborne microorganisms via disturbances to the leaf surface, precipitation or rain splash, and soil pollution (Redford et al., 2009).

The Mantel test found that elevation, distance from water, tree diameter at breast height, mean height of bamboo, mean base diameter of bamboo, tree height, shrub coverage and the number of shrubs, all significantly affected phyllosphere microbial community (Table S5). Jackson and colleagues (2006) found that the changes in the phyllosphere bacterial community in resurgent ferns were related to rainfall and humidity. Similarly, Laforest and colleagues (2016) found that the host species, habitat and climate (average summer temperature and precipitation) drove the phyllosphere bacterial community structure in temperate trees. In this study, elevation had the strongest relationship with phyllosphere microbial community. Elevation was significantly and positively correlated to many bacteria phyla abundance, such as *Proteobacteria* (Fig. 5). However, with an increase in elevation, both the Shannon and Sobs indices declined (Fig. S6). Higher elevations generally have fewer and more widely distributed water sources, and lower temperatures can limit the fluidity of microbial cell membranes and proteins, which are not conducive to microbial reproduction and growth (Zhang et al., 2014).

Changes to the phyllosphere microbial community could impact the degradation and absorption of plant nutrients and the metabolism of enzymes (Fazal et al., 2021). In this study, we used PICRUSt to predict the function of phyllosphere bacteria of three bamboo species foraged by giant pandas. Our data shows that the gene function spectrum of phyllosphere bacteria of *F. ferax* was significantly different from the other two bamboo species. The relative abundance of gene types in the membrane transport secretion system, signal transduction and oxidative phosphorylation metabolism in the third level of the KEGG pathway for *F. ferax* were lower than for the other two bamboo species (Fig. 6). This indicated that the low-altitude *F. ferax* may need less energy and protein to maintain the physiological activities of some phyllosphere bacteria. While the high-altitude *A. spanostachya* and *Y. lineolate* may require more material and energy to adapt to the colder, lower oxygen environment. It is worth noting that the relative abundance of gene types in transporters and ABC transporters were the most expressed pathways in membrane transport at level 2. Hamana and colleagues (2012) revealed that ABC transporters can protect animals from the barrier of toxic substances. In addition, genes related to replication and repair may help reduce damage to biomolecules and may help bamboo adapt to high altitude environments. However, our results are only based on predicted metagenomics, and do not represent the actual function of leaf-peripheral bacteria.

Food microbes can affect the gut microbes of animals (Kohl and Dearing 2014;Kohl et al. 2016). Lei and colleagues (2020) found significant associations of certain bacteria and fungi between bamboo and the gut of giant panda. The diversity of bamboo bacteria was also positively correlated with that of gut bacteria in giant panda. Giant pandas prefer to consume bamboo that grows naturally at high altitudes, probably because the total number of endophytic bacteria in high altitudes tends to be lower (Helander et al., 2013). There's a lot of work needed to fully understand the relationship between the food microbes and gut microbes of pandas. To explore the relevance of these genes in the environmental adaptability of giant pandas and bamboo, further research is needed to directly sequence the metagenomics of the phyllosphere microbial community and determine whether there are specific enzymes related to digestion in giant pandas.

Conclusions

In this study, high-throughput sequencing was used to explore the factors that influence phyllosphere bacterial composition in three bamboo species (*A. spanostachya*, *Y. lineolate* and *F. ferax*) foraged by giant panda in different seasons (spring vs. autumn), in Liziping National Nature Reserve (Liziping NR), China. Our findings suggested that the diversity of *F. ferax* phyllosphere bacterial species was greater than that of the other two bamboo species in both seasons, indicating that low altitude was a promoter of bamboo phyllosphere microbial richness and diversity. Besides, phyllosphere bacterial diversity was also significantly higher in autumn than in spring, implying that season-related changes in environmental factors (e.g.,temperature and moisture) may influence bacterial communities. In autumn, giant pandas prefer bamboo at higher altitudes, which has lower abundance and diversity of phyllosphere bacteria. Lower abundance and diversity of phyllosphere bacteria also were found at lower altitudes in spring than in autumn. Therefor, giant pandas prefer to lower abundance and diversity of phyllosphere bacteria may attribute to nutrient-rich bamboo and adapted climate, these may be also considered as panda adaptation to partially meet the higher energy requirements needed for survival in the harsh cold and hypoxic environment. All these findings could provide a reference for the restoration and management of giant panda habitat and food resources in this area, especially for those small isolated populations of giant pandas in Xiaoxiangling mountains.

Declarations

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Authors' contributions

Conceived and designed the study: MS H, ZJ Z, JJ L. Collected data and samples in the field: JJ L, L W, JM X, Y Y, J W, LW K, Y L. Processed samples in the lab: MS H, JJ L, JM X, Y Y, J W. Analysed the data: MS H, JJ L, Y Y. Wrote the paper: MS H, ZJ Z, JJ L, L W. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

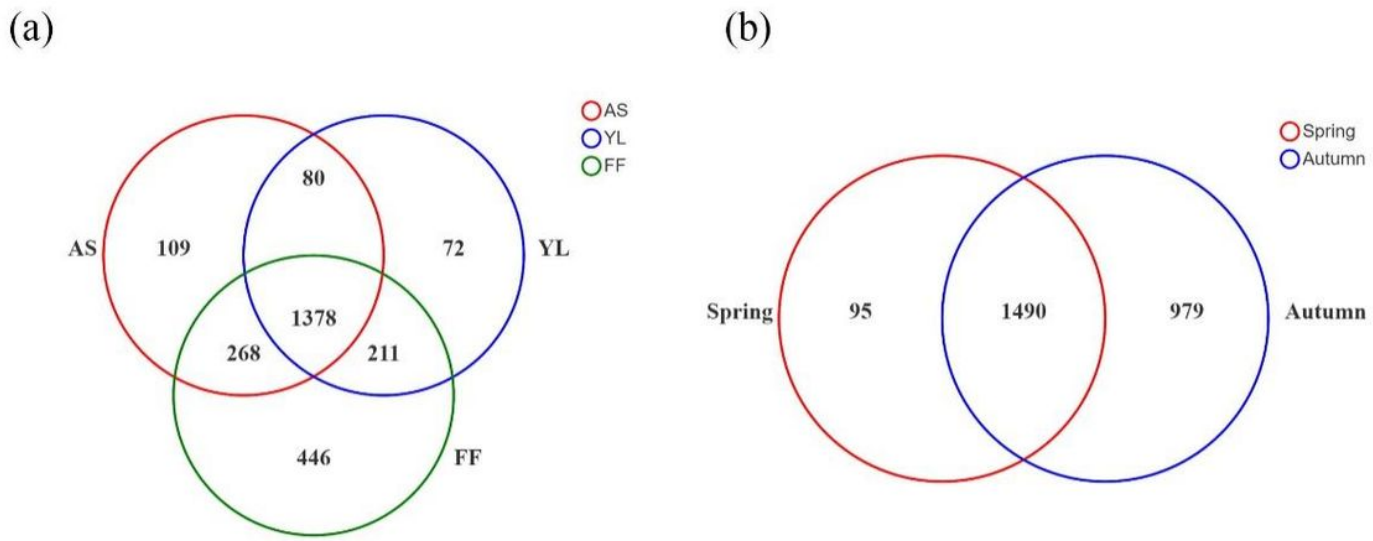

Figure 1

Venn Diagram showing overlap/non-overlap of phyllosphere bacteria OTUs among three bamboo species (a) and two seasons (b). (AS: A. spanostachya; YL: Y. lineolate; FF: F. ferax)

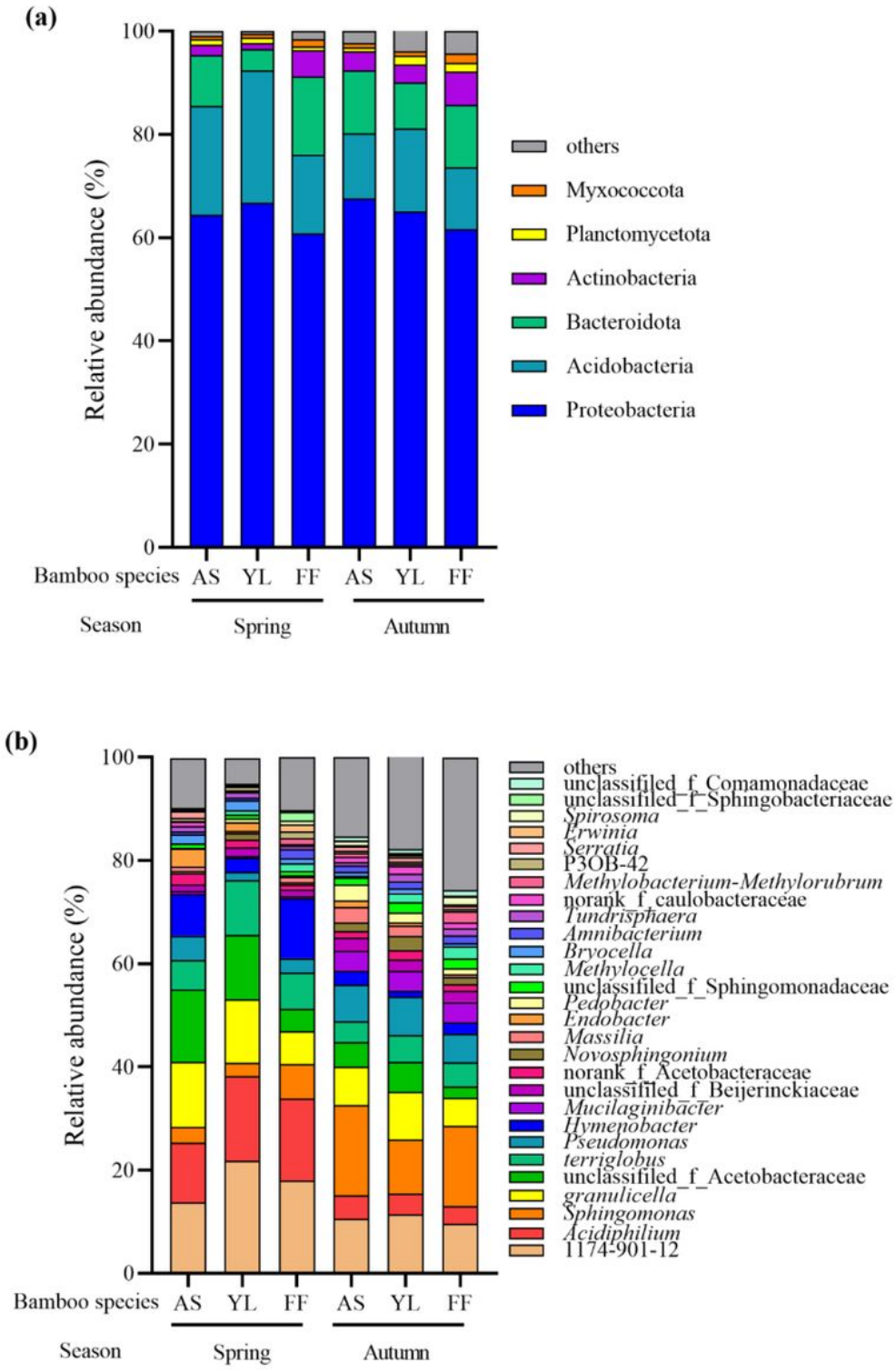

Figure 2

Relative abundance of different phyllosphere bacterial phyla (a) and genus (b) among A. spanostachya (AS), Y. lineolate (YL) and F. ferax (FF) in spring and autumn.

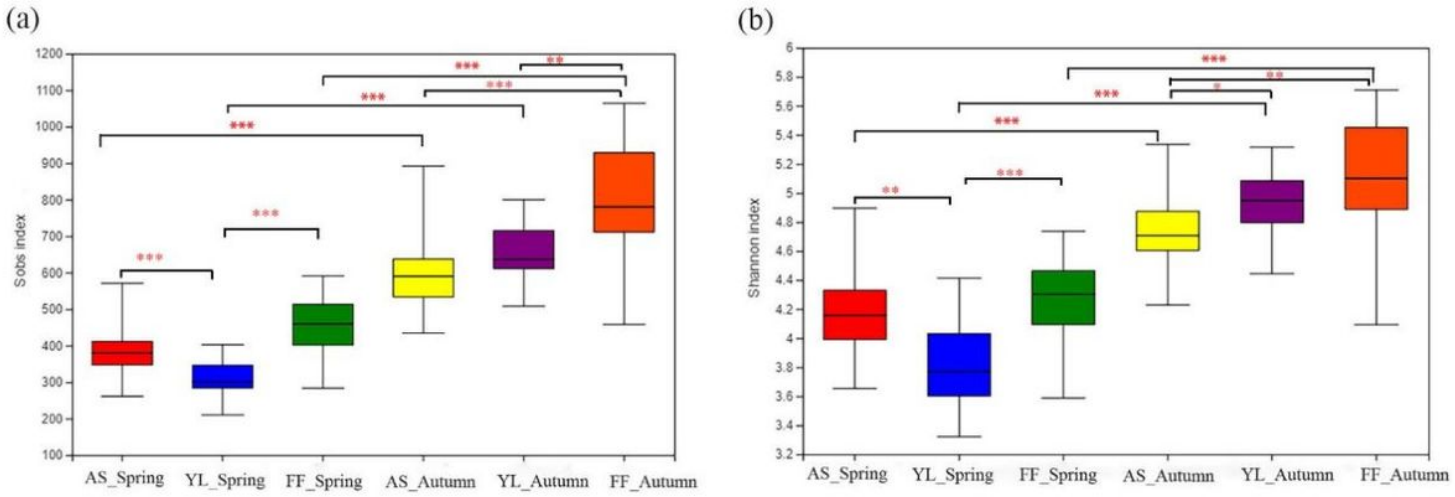

Figure 3

The observed species index (Sobs index) (a) and Shannon index (b) of phyllosphere bacterial community among A. spanostachya (AS), Y. lineolate (YL) and F. ferax (FF) in spring and autumn. Student's t test was used to test the significance. (*0.01<P<0.05, ** 0.001<P<0.01, *** P<0.001)

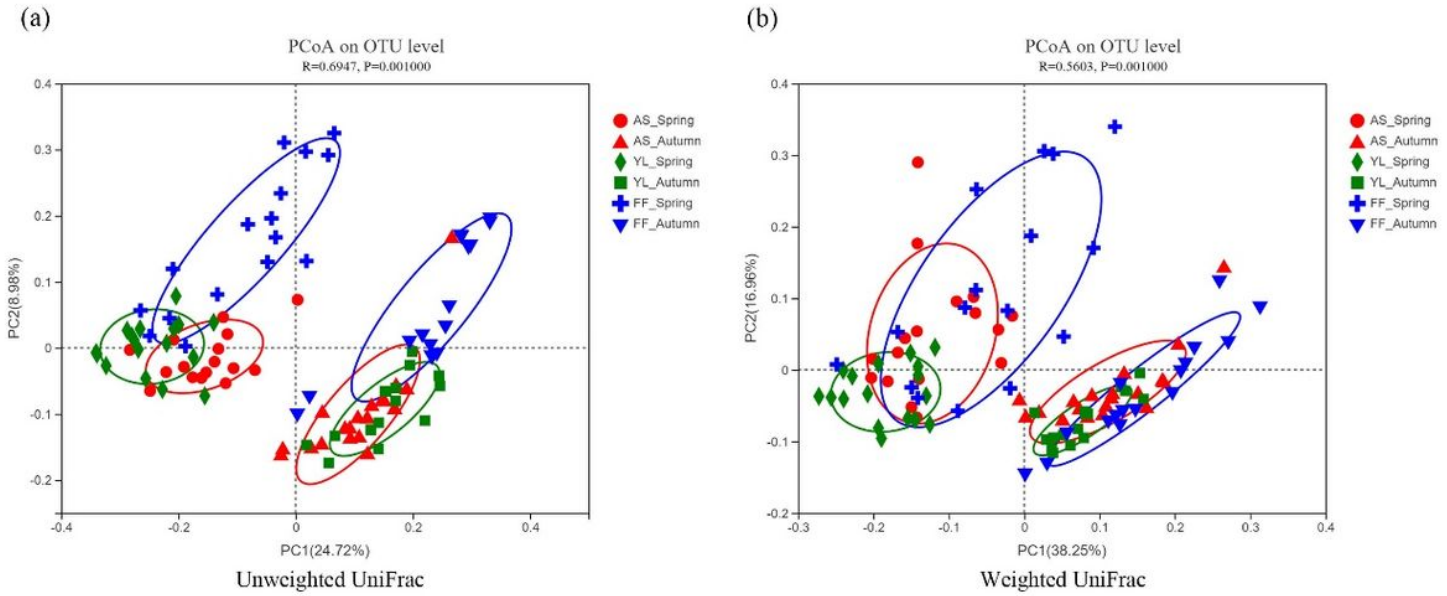

Figure 4

Principal Coordinate Analysis (PCoA) plots of phyllosphere bacterial communities using the unweighted UniFrac method (a) and weighted UniFrac method (b) at OTU level among A. spanostachya (AS), Y. lineolate (YL) and F. ferax (FF) in spring and autumn.

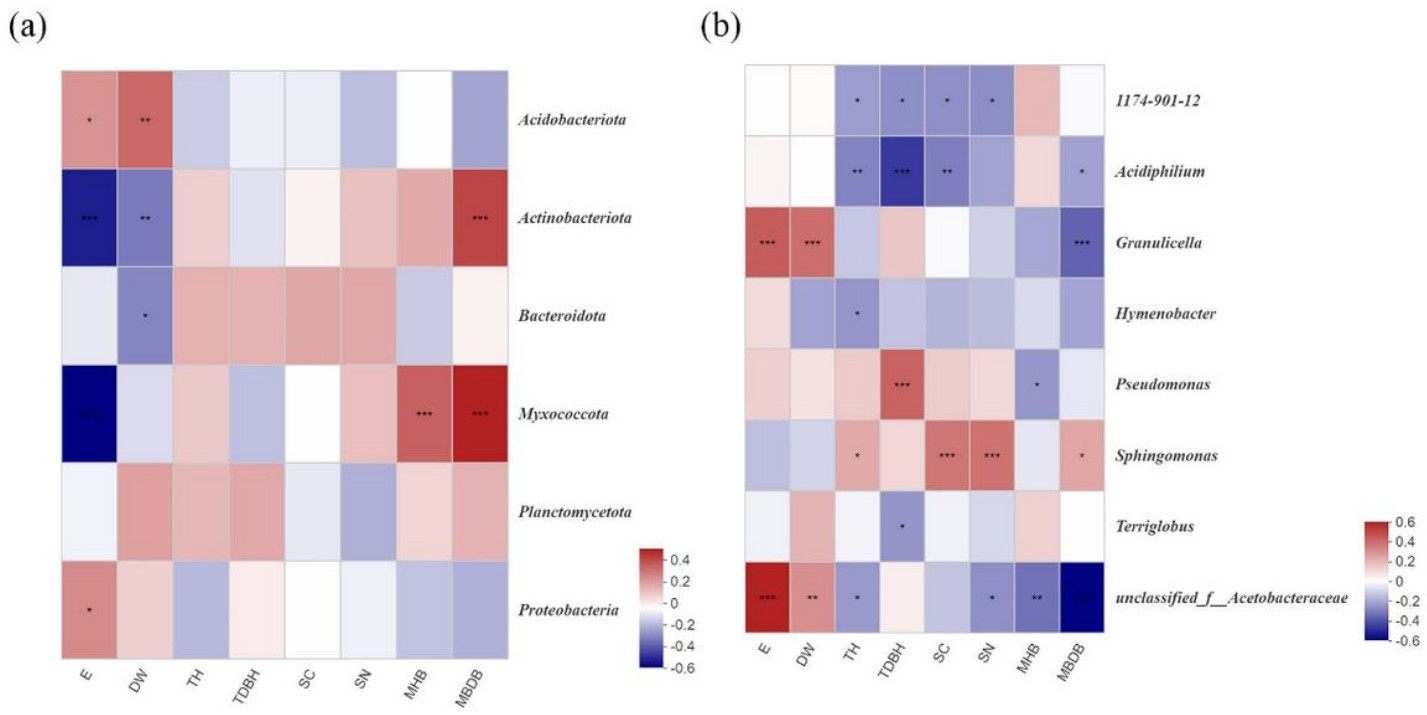

Figure 5

Correlation heatmap of bamboo environmental factors and read numbers at the phylum (a) and genus (b) level for bacteria. E: elevation; DW: distance from water; TH: trees height; TDBH: trees diameter at breast height; SC: shrubs coverage; SN: shrubs numbers; MHB: mean height of bamboo; MBDB: mean base diameter of bamboo. The color intensity in each panel indicates the relative correlation between bamboo environmental factors and read numbers of each group. (*0.01<P<0.05, ** 0.001<P<0.01, *** P<0.001)

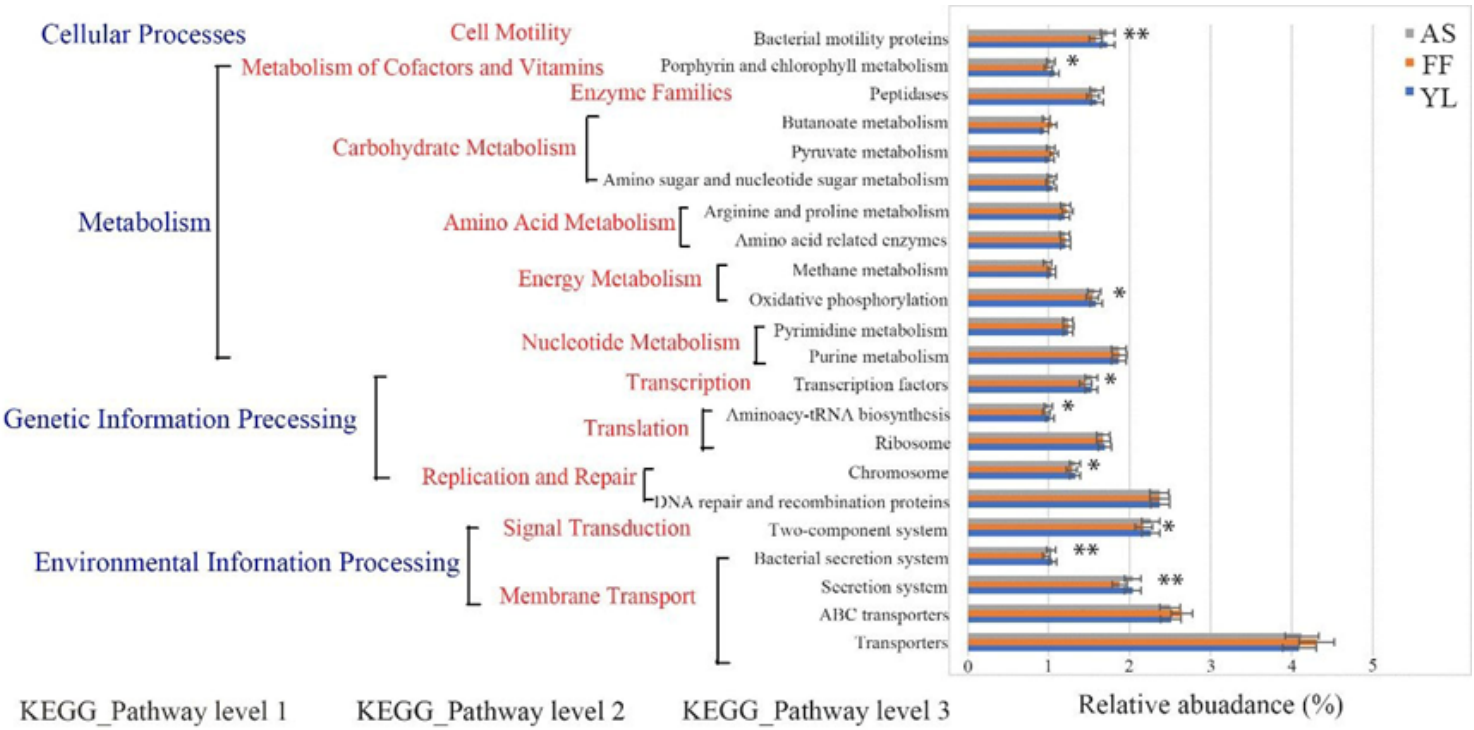

Figure 6

Functional predictions for phyllosphere bacterial communities with significantly different KEGG pathways (P< 0.05) for the three bamboo species (AS, FF, and YL). KEGG pathways at Level 1, Level 2, and Level 3 are represented. “*” and “**” indicate the significance level at0.05 and 0.01, respectively

Supplementary Files

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- TableS7Functionalpredictions.xlsx