The effect of *Epichloë* endophyte on phyllosphere microbes and leaf metabolites in *Achnatherum inebrians*
The effect of *Epichloë* endophyte on phyllosphere microbes and leaf metabolites in *Achnatherum inebrians*

Bowen Liu,1 Yawen Ju,1 Chao Xia,1 Rui Zhong,1,* Michael J. Christensen,2 Xingxu Zhang,1,3,* and Zhibiao Nan1

**SUMMARY**

Upon exposure to the prevailing environment, leaves become increasingly colonized by fungi and bacteria located on the surface (epiphytic) or within (endophytic) the leaves. Many cool season grasses, including *Achnatherum inebrians*, host a seed-borne, intercellular, mutualistic *Epichloë* fungal endophyte, the growth of which is synchronized with the host grass. A study utilizing illumina sequencing was used to examine the epiphytic and endophytic microbial communities in *Epichloë* endophyte-infected and endophyte-free *A. inebrians* plants growing under hot dry field conditions. The presence of *Epichloë* endophyte increased the Shannon and decreased Simpson diversity of bacterial and fungal communities. *Sphingomonas* and *Hymenobacter* bacteria and *Filobasidium* and *Mycosphaerella* fungi were growing largely epiphytically, whereas *Methylobacterium*, *Escherichia-Shigella*, and the fungus *Blumeria* were mostly found within leaves with the location of colonization influenced by the *Epichloë* endophyte. In addition, leaf metabolites in *Epichloë*-infected and *Epichloë*-free leaves were examined using LC/MS. *Epichloë* was significantly correlated with 132 metabolites.

**INTRODUCTION**

The phyllosphere, composed of the aerial parts of plants and dominated by leaves, covering approximately 640 million km², is a universal and vital habitat for bacteria and fungi that includes endophytes inside of leaves and superficial epiphytes (Santamaria and Bayman, 2005; Vorholt, 2012). In light of both nutrient concentration (Mercier and Lindow, 2000) and topography (Mechaber et al., 1996), the phyllosphere is a highly heterogeneous and extensive environment (Andrews and Harris, 2003), which provides many ecological niches for microbial colonization. Phyllosphere bacteria and fungi, which have high species diversity, are significant parts of the microbial community and play important roles in ecosystem functions (Arnold et al., 2007; Partida-Martínez and Heil, 2011). Leaf-associated bacteria and fungi represent ancient and widespread symbiotic relationships (Arnold et al., 2003; Partida-Martínez and Heil, 2011). They can affect the growth and function of the host plants in many ways, including producing growth-promoting nutrients and hormones (Gourion et al., 2006) and enhancing the host plants resistance to biotic and abiotic stresses (Innerebner et al., 2011; Xia et al., 2018). For example, bioactive molecules produced by *Pseudomonas syringae* can induce stomatal closure, thereby affecting the entry of pathogens into the apoplast (Melotto et al., 2006). In addition, phyllosphere bacteria and fungi are prominent parts in the carbon-nitrogen cycle of ecosystems (Whipps et al., 2010; Purahong and Hyde, 2011; Peñuelas and Terradas, 2014). They have potential impacts on plant biogeography and ecosystem function through regulating host performance in differing environmental conditions (Friesen et al., 2011; Guerreiro et al., 2018).

Metabolites represent the physiological status of plant organisms at the metabolic level. They are the ultimate result of gene transcription and protein expression and the material basis of the phenotype of the organisms (Matsuda et al., 2012; Jin et al., 2017; Cao et al., 2019). Meanwhile, metabolites can affect or regulate transcription and expression of genes and activity of proteins (Alcázar et al., 2011; Agati et al., 2012). Internal and external factors, such as plant growth and environmental factors, will change metabolite concentration or metabolic flow (Bowne et al., 2012; Dong et al., 2015; Nam et al., 2016), and these changes involve multiple metabolites and metabolic pathways (Scandiani et al., 2015). For example, under the condition of water deficit, the improvement of frost resistance of alfalfa (*Medicago sativa* L.) was related to the increase of soluble sugar, amino acid, lipid, and lipid molecular content (Xu et al., 2020). At present, many studies have integrated plant metabolomics...
and other omics, such as transcriptomics (Ma et al., 2016), genomics (Chen et al., 2014), and proteomics (Li et al., 2020), thereby opening up new opportunities for the studies of plant metabolic pathways, genetic structure, and functional gene identification. Studies on the correlation between metabolomics and microbiomics mainly focused on the gut microbiome (Liu et al., 2017; Franzosa et al., 2019) and fecal microbiome (Schmidt et al., 2018) but few on plant tissues. Previous studies on rhizosphere metabolites showed that rhizosphere soil metabolites influenced the rhizosphere microbiome (Massalha et al., 2017; Wen et al., 2020).

Epichloë endophytes have been found in many cool-season grasses (Schardl et al., 2005; Kulda and Bacon, 2008; Leuchtmann et al., 2014). They asymptotically colonize in all tissues of host grasses except the roots (Christensen et al., 2008); besides, for many Epichloë species, transmission is entirely vertical, in seed produced by host plants (Schardl et al., 2005). The relationships between Epichloë endophytes and their hosts are generally regarded to be mutualistic (Müller and Krauss, 2005). Most studies are focused on the symbiosis between Epichloë endophytes and Lolium and Festuca species, and the presence of Epichloë endophytes can improve the persistence and productivity of host plants (Johnson et al., 2013; Oberhofer et al., 2014; Soto-Barajas et al., 2016; Bastias et al., 2017).

Achnatherum inebrians (drunken horse grass, DHG), a perennial bunchgrass, is widely distributed in alpine and subalpine grasslands of Gansu, Inner Mongolia, Xinjiang, Qinghai, and Tibet in China (Li et al., 2004). Almost every A. inebrians plant was found in surveys to be infected by either E. gansuensis (Li et al., 2004) or E. inebrians (Chen et al., 2015). Alkaloids, including ergonovine and ergine, are present in DHG plants infected with Epichloë endophytes (Zhang et al., 2014) and can be toxic to livestock and deter ingestion (Liang et al., 2017).

Epichloë endophytes can improve the resistance and adaptability of DHG under adverse conditions, such as elevated levels of salt (Li et al., 2018), heavy metals (Zhang et al., 2010), drought (Xia et al., 2018), low temperature (Chen et al., 2016), infection by plant pathogens, in particular Blumeria graminis (Xia et al., 2015, 2016), and insect pests (Zhang et al., 2012). The effects of Epichloë endophytes on plant-associated microbes, both belowground and aboveground, have been reported in different host species and environments (Robert and Ferraro, 2015; Rojas et al., 2016; Bell-Dereske et al., 2017). Previous studies reported that Epichloë endophytes can affect rhizosphere and phyllosphere microbial community structures in Festuca arundinacea (tall fescue) (Robert and Lindow, 2014; Roberts and Ferraro, 2015). In addition, Maria et al. (2020) found that Epichloë endophytes modify the foliar anatomy of L. multiflorum, the anatomical characteristics are the result of plants adapting to different environments. Our previous studies involving A. inebrians found that the presence of Epichloë endophytes in the A. inebrians plants decreased the root-associated fungal community diversity under cultivation (Zhong et al., 2018) and increased root-associated AMF diversity under drought conditions but decreased root-associated AMF diversity under the water addition treatment (Zhong et al., 2021). In addition, Epichloë endophytes in A. inebrians significantly decreased the diversity of the root-associated bacterial community but increased the diversity of the rhizosphere soil bacterial community (Ju et al., 2020). The mechanisms by which the presence of an Epichloë endophyte can affect above ground microbes including some plant pathogenic fungi affecting leaves is not understood. One possibility is that induced changes in the bacterial and fungal communities associated with leaves may alter the infectivity of some pathogenic fungi, reducing the incidence of disease. Another possible factor could be the presence of the endophyte results in the accumulation of some antifungal products, either secondary metabolites of the endophyte or plant metabolites induced by the presence of the endophyte. Bioassay studies have revealed that some Epichloë endophytes produce antifungal substances both when growing saprophytically in vitro and also when growing biotrophically in host plants (Christensen, 1996). Therefore, we conducted a study to look at how the presence of an Epichloë endophyte affected the bacterial and fungal communities, both epiphytic and endophytic, of field-growing A. inebrians plants and also metabolites of leaves. In this study, we focused solely on the plant’s response metabolically to the presence of certain taxa.

We hypothesized that

1. Epichloë endophytes would alter the endophytic and epiphytic phyllosphere microbial communities.
2. Epichloë endophytes can influence the content and classes of leaf metabolites.
3. There are complex and close correlations between phyllosphere microbes and leaf metabolites.
RESULTS
Phyllosphere bacterial and fungal communities

The minimum, maximum, and average sequence numbers of bacteria obtained from each leaf sample by the high-throughput sequencing were 27,897, 98,417, and 54,612 and the minimum, maximum, and average sequence numbers of fungi were 31,824, 360,356, and 119,607, respectively (Table S1). These sequences were normalized with the minimum sequence number and then classified into 704 bacterial and 479 fungal operational taxonomic units (OTUs) at a 97% sequence similarity cutoff, respectively (Figures S1, S3, and S4). In this study, according to the infection status of the Epichloë endophyte and the endophytic and epiphytic environment of the phyllosphere, the phyllosphere microbial communities were defined as four groups - these were Enl (endophytic phyllosphere microbial community of endophyte-infected plants), Epi (epiphytic phyllosphere microbial community of endophyte-infected plants), EnF (endophytic phyllosphere microbial community of endophyte-free plants), and EpF (epiphytic phyllosphere microbial community of endophyte-free plants). The numbers of shared OTUs were analyzed among treatments (Figure S1). When the 652 endophytic and 609 epiphytic bacterial OTUs of the phyllosphere of endophyte-infected plants were combined, it was found that 560 OTUs were shared between the two groups; 542 and 512 OTUs were further shared with the endophytic and epiphytic bacteria of endophyte-free plants, respectively (Figure S1A). Among the Enl, Epi, EnF, and EpF bacterial communities, 128, 41, 64, and 18 OTUs were shared by all eight samples, respectively (Figures S2A–S2D). Samples EnF6, EpF6, En7, and Epi7 had the largest number of unique OTUs; 30, 64, 18, and 32 respectively (Figures S2A–S2D). The Enl fungal community contained 465 OTUs, of which 301, 462, and 306 OTUs were shared with the EnF, Epi, and EpF fungal community, respectively (Figure S1B). Only one unique OTU was in the Enl fungal community, and no unique OTUs were detected in the other three fungal communities (Figure S1B). In addition, in the Enl, Epi, EnF, and EpF fungal communities, 45, 84, 34, and 76 OTUs were shared by all eight samples, respectively (Figures S2E–S2H).

The relative abundance of main groups of phyllosphere bacterial and fungal communities at the phylum and genus level was different among treatments (Figures 1 and 2). Most phyllosphere bacteria belonged to the four major phyla of Proteobacteria (54.42%), Firmicutes (29.92%), Actinobacteria (6.31%), and Bacteroidetes (6.19%) (Figure 1A and Table S2). The relative abundance of Actinobacteria in the Epl bacterial community was significantly (p < 0.05) higher than that in Enl and EnF bacterial communities, whereas there was no significant difference with that in the EpF bacterial community (Table S2). The relative abundance of the other three phyla had no significant differences among the four treatments (Table S2). Of the 269 bacterial genera identified, the 9 most abundantly represented were Exiguobacterium (12.48%), Methyllobacterium (9.78%), Pseudomonas (8.46%), Sphingomonas (8.43%), Escherichia-Shigella (3.62%), Providencia (3.31%), Acinetobacter (3.24%), Hymenobacter (3.24%), and Erwinia (3.17%) (Figure 2A and Table 1). Among them, the relative abundance of Sphingomonas and Hymenobacter in the epiphytic bacterial community was significantly (p < 0.05) higher than that in the endophytic bacterial community (Enl and EnF) (Figure 2A and Table 1).

In addition, in the phyllosphere fungal community, the most abundant phylum was Ascomycota (58.62%) (Figure 1B and Table S2). The next most abundant phyla were Basidiomycota (24.49%) and Mortierellomycota (2.64%) (Figure 1B and Table S2). Basidiomycetes were significantly (p < 0.05) more abundant in the Epl fungal community than in Enl and EnF fungal communities, but there was no significant difference with that in the EpF fungal community (Table S2). Meanwhile, the relative abundance of Ascomycota and Mortierellomycota had no significant difference among these fungal communities (Table S2). The classification of fungal OTUs at the genus level found that in the endophytic fungal community the most abundant OTUs belonged to Blumeria (11.45%) and Alternaria (11.03%) (Figure 2B and Table 1). By contrast, in the epiphytic fungal community, the dominant OTUs were identified as Filobasidium (21.87%) and Mycosphaerella (14.64%) (Figure 2B and Table 1). Moreover, the relative abundance of Epichloë and Phaeosphaeria in the Enl community was significantly (p < 0.05) higher than that in the other three communities (Figure 2B and Table 1).

Phyllosphere bacterial and fungal community diversities

The Shannon diversity, Chao, and ACE richness indexes of endophytic bacteria were significantly (p < 0.05) higher than those of epiphytic bacteria, whereas the Simpson index was lower than that of epiphytic bacteria (Figures 3A–3D). Meanwhile, the presence of Epichloë endophyte increased the Shannon, Chao, and ACE indexes, but decreased the Simpson index of the phyllosphere bacterial community in A. inebrians (Figures 3A–3D).
The Shannon and Simpson diversity indexes of endophytic fungi were not significantly different from those of epiphytic fungi, but the Chao and ACE richness indexes were significantly (p < 0.05) higher than those of epiphytic fungi (Figures 3E–3H). Furthermore, *Epichloë* endophyte increased Shannon diversity and decreased Simpson diversity in the fungal community of *A. inebrians* (Figures 3E and 3F).

The nonmetric multidimensional scaling (NMDS) ordination revealed that the community diversities of phyllosphere endophytic microbes (included bacteria and fungi) were significantly (p = 0.0001) different from epiphytic microbes (Figure 4 and Table 2).
The effect of *Epichloë* endophytes on leaf metabolites

A total of 414 detected metabolites were annotated in endophyte-infected (EI) and endophyte-free (EF) leaves of *A. inebrians* (Table S3). We performed the orthogonal projections to latent structures-discriminant (OPLS-DA) and principal component analysis (PCA) analysis on these metabolites (Figure S5). The OPLS-DA model (R2X = 0.873, R2Y = 0.968, Q2Y = 0.881) was stable and effective, which indicated there were obvious differences of the metabolites between EI and EF leaves (Figure S5A). PCA results (PC1 58.9%, PC2 11.7%) showed that the content of detected metabolites significantly varied under different endophyte treatments (Figure S5B).
endophyte (Table S4). However, differential metabolites of 12 classes, including lipids-glycerophospholipids and sphingolipids, phenols, nucleosides and their derivatives, terpenoids, vitamins, antibiotics, curcuminoids, steroid hormones, flavones, proteins-enzymes, and transport proteins, were downregulated (Table S4).

Based on the FC (fold change) > 2, VIP>1 and p < 0.05, the 132 differential metabolites between EI and EF were separated (Table S4). Among these, there were 21 upregulated differential metabolites and 111 downregulated differential metabolites (Figure S6 and Table S4).

The 132 differential metabolites belonged to 24 classes. According to the number of differential metabolites in each class, the 6 major classes included amino acids and their derivatives, organic acids, carboxylic acid derivatives, carbohydrates and their derivatives, organonitrogen compounds, and lipids-glycerophospholipids (Table 3).

Purine derivatives, indole derivatives, and phytoestrogens were upregulated with the infection of *Epichloë* endophyte (Table S4). However, differential metabolites of 12 classes, including lipids-glycerophospholipids and sphingolipids, phenols, nucleosides and their derivatives, terpenoids, vitamins, antibiotics, curcuminoids, steroid hormones, flavones, proteins-enzymes, and transport proteins, were downregulated (Table S4).

We selected the top10 upregulated and downregulated differential metabolites based on Log2FC in the EI as compared to the EF leaves (Table 4). Among these, enterodiol (phytoestrogens), 3-indoleacetonitrile (indole derivatives), viloxazine (ethers), and suberic acid (lipids-fatty acids) were significantly (p < 0.001)
Figure 3. Phyllosphere microbial community alpha diversity index

Endophytic and epiphytic (A, B, C, and D) bacteria and (E, F, G, and H) fungi alpha diversity index of endophyte-infected and endophyte-free leaves. In box plots, the full line represents the median and the dotted line represents the mean, box edges show the 75th and 25th percentiles, and whiskers extend to 1.5× the interquartile range. Two-way ANOVA (n = 8) (EI: endophyte-infected, EF: endophyte-free) (P: endophytic and epiphytic environment of phyllosphere, E: *Epichloë* endophyte infection status).
upregulated metabolites (Table 4). Zotepine (organonitrogen compounds), diosmetin (flavones), vanillin (phenols), 9-Decen-1-ol (Carboxylic Acid Derivatives), and trans-ferulic acid (organic acids) were significantly (p < 0.001) downregulated metabolites (Table 4). In addition, for alkaloids, the normalized abundance of methylergonovine was significantly (p < 0.001) upregulated in EI leaves (Table 4).

A total of 42 differential metabolites were annotated with KEGG (Kyoto Encyclopedia of Genes and Genomes) database and 20 metabolic pathways were significantly enriched (p < 0.05) (Figure S7). KEGG analysis showed that phenylalanine metabolism and galactose metabolism were major enriched pathways (Figure S7).

Correlations between phyllosphere microbes and metabolites

To explore the complex interaction between phyllosphere microbes and leaf metabolites in response to infection of *Epichloë* endophyte in *A. inebrians*, we performed the correlation analysis between microbiome and metabolome, focusing on representative differential metabolites and relatively abundant microbial phyla. The correlation heat map revealed a total of 229 significant (p < 0.05) correlations between differential metabolites and microbial phyla (Figure 5).

There were 115 significant (p < 0.05) correlations between 61 differential metabolites and 9 endophytic bacterial phyla (Figure 5A and Table S5). The bacterial phyla included Actinobacteria, Cyanobacteria, Firmicutes, and Fusobacteria. The relative abundance of Actinobacteria, Firmicutes, and Fusobacteria was

---

**Table 2. The statistical test of similarity (ANOSIM) and permutational multivariate two-way analysis of variance (PERMANOVA)**

| Type | Treatment | Degrees of freedom | Bray-Curtis | Bray-Curtis |
|------|-----------|--------------------|-------------|-------------|
|      |           |                    | F   | P       | R   | P       |
| Bacteria | P   | 1                  | 9.4132 | 0.0001 | 0.71749 | 0.0001 |
|        | E   | 1                  | 1.5132 | 0.1308 | 0.049526 | 0.1791 |
|        | P*E | 1                  | 1.4257 | 0.1671 |          |        |
| Fungi  | P   | 1                  | 10.224 | 0.0001 | 0.50739 | 0.0001 |
|        | E   | 1                  | 1.4445 | 0.1867 | 0.048549 | 0.2069 |
|        | P*E | 1                  | 1.0481 | 0.368  |          |        |

P: endophytic and epiphytic environment of phyllosphere, E: *Epichloë* endophyte infection status.
increased in EI, but Cyanobacteria abundance was decreased in EI (Table S2). In the endophytic bacterial community, only OTU3631 and OTU7 represented Cyanobacteria, and they were positively correlated with enterodiol and negatively correlated with diosmetin and sphingosine (Figures 5A, Table S5, S14, and S16). Firmicutes and Fusobacteria showed positive correlations with indoleacetic acid and suberic acid and negative correlations with vanillin and rosmarinic acid (Figure 5A and Table S5). In addition, there was positive correlation between 169 OTUs of Firmicutes and deguelin(-) (Figure 5A, Tables S5, S14, and S16). The OTUs (OTU110, OTU119, and OTU339) representing Fusobacteria in endophytic bacteria had positive correlation with dexoxycoformycin and negative correlation with coniferol and traumatic acid (Figure 5A, Tables S5, S14, and S16). The correlation analysis at the genus level revealed that *Pseudomonas* was negatively correlated with indoleacetic acid, and the relative abundance of this genus in EnI was lower than that in EnF (Tables 1 and S10). Enterodiol, indoleacetic acid, suberic acid, deguelin(-), and dexoxycoformycin were significantly (p < 0.05) upregulated in EI, whereas the content of diosmetin, sphingosine, vanillin, rosmarinic acid, coniferol, and traumatic acid was significantly (p < 0.05) lower than that in EF (Table S4).

There were 50 significant (p < 0.05) correlations between 50 differential metabolites and 10 epiphytic bacterial phyla (Figure 5B and Table S6). Only Actinobacteria had significant (p < 0.05) correlations with differential metabolites (Figure 5B and Table S6). The relative abundance of Actinobacteria in EI was higher than that in EF (Table S2). Enterodiol, suberic acid, deguelin(-), and dexoxycoformycin were positively correlated with 50, 44, 35, and 44 OTUs of Actinobacteria, respectively (Figure 5B, Tables S6, S14, and S16). 38, 44, 46, 50, and 39 OTUs of Actinobacteria exhibited negative correlations with benztrpine, aldicarb, coniferol, diosmetin, and vanillin (Figure 5B, Tables S6, S14, and S16). *Sphingomonas*, whose relative abundance in the epiphytic bacterial community was significantly (p < 0.05) higher than that in endophytic bacterial community, was significantly (p < 0.05) positively correlated with enterodiol and negatively correlated with sphingosine, phytosphingosine, and metaraminol (Tables 1 and S11). The content of benztrpine, aldicarb, phytosphingosine, and metaraminol were downregulated under *Epichloë* endophyte infection (Table S4).

There were 39 significant (p < 0.05) correlations between 34 differential metabolites and 8 endophytic fungal phyla (Figure 5C and Table S7). These correlations are mainly related to Ascomycota, Mucoromycota, and Zoopagomycota. The relative abundance of Mucoromycota and Zoopagomycota in EI was higher than that in EF, but the relative abundance of Ascomycota was lower than that in EF (Table S2). Mucoromycota (OTU153) and Zoopagomycota (OTU236 and OTU85) had positive correlation with deguelin(-) and negative correlations with jasmine lactone and bisdemethoxycurcumin (Figure 5C, Tables S7, S15, and S17). In addition, vanillin, benztrpine, and rosmarinic acid were negatively correlated with Zoopagomycota (Figure 5C and Table S7). Besides, there was a negative correlation between indoleacetic acid and 87 OTUs of Ascomycota (Figure 5C, Tables S7, S15, and S17). *Phaeosphaeria*, belonging to Ascomycetes, was significantly (p < 0.05) positively correlated with methylergonovine and negatively correlated with

| Table 3. The summary classification of the 132 differential metabolites |
|--------------------------|--------------------------|
| Class                        | Number of metabolites | Class                      | Number of metabolites |
| Amino acids and their derivatives | 32                        | Alkaloids                  | 3                      |
| Organic acids               | 18                        | Vitamins                   | 3                      |
| Carboxylic acid derivatives | 12                        | Antibiotics                | 2                      |
| Carbohydrates and their derivatives | 9                        | Curcumins                  | 2                      |
| Organonitrogen compounds    | 7                         | Lipids-sphingolipids       | 2                      |
| Lipids-glycerophospholipids | 7                         | Ketones                    | 1                      |
| Lipids-fatty acids          | 5                         | Steroid hormones           | 2                      |
| Phenols                     | 4                         | Indole derivatives         | 2                      |
| Nucleosides and their derivatives | 4                        | Flavones                   | 3                      |
| Ethers                      | 4                         | Phytoestrogens             | 1                      |
| Terpenoids                  | 4                         | Proteins-enzymes           | 1                      |
| Purine derivatives          | 3                         | Proteins-transport Proteins| 1                      |

The left and right panels are the same column split in two.
jasmine lactone, Glycerol 3-phosphate, and tobramycin (Table S12). The relative abundance of Phaeosphaeria in EnI was significantly (p < 0.05) higher than that in other fungal communities (Table 1). Jasmine lactone, bisdemethoxycurcumin, Glycerol 3-phosphate, and tobramycin were significantly (p < 0.05) downregulated in EI, whereas methylergonovine was significantly (p < 0.05) upregulated (Table S4). In addition, only OTU6 represented in EnI was significantly (p < 0.05) higher than that in other fungal communities (Table 1). Jasmine lactone, bisdemethoxycurcumin, Glycerol 3-phosphate, and tobramycin (Table S12). The relative abundance of methylergonovine was significantly (p < 0.05) negatively correlated with methylergonovine, and 95 OTUs of Basidiomycota (Figure 5D and Table S8). Methyl 4-hydroxybenzoate was positively correlated with 33 OTUs of Ascomycota, whereas these correlations; the relative abundance of Ascomycota decreased, but Basidiomycota was increased in EnI. In particular, we carried out the correlation analysis of the phyllosphere microbial communities, and Spearman’s rank correlation analysis found that Epichloë was significantly (p < 0.05) correlated with 132 detected differential metabolites between EI and EF leaves (Tables S9 and S17). Among these, 21 upregulated metabolites were positively correlated with Epichloë, and 111 downregulated differential metabolites were negatively correlated (Table S9).

There were 25 significant (p < 0.05) correlations between 24 differential metabolites and 8 epiphytic fungal phyla (Figure 5D and Table S8). Ascomycota and Basidiomycota were relatively abundant phyla involved in these correlations; the relative abundance of Ascomycota decreased, but Basidiomycota was increased in EI (Table S2). Methyl 4-hydroxybenzoate was positively correlated with 33 OTUs of Ascomycota, whereas negatively correlated with 95 OTUs of Basidiomycota (Figure 5D and Table S8). Methyl 4-hydroxybenzoate was significantly (p < 0.05) downregulated in EI as compared to the EF (Table S4). For genera enriched in epiphytic fungal communities, Blumeria and Neosetophoma were positively correlated with jasmine lactone and negatively correlated with deguelin(-) (Table S13). In addition, Neosetophoma was also significantly (p < 0.05) negatively correlated with methylergonovine, and Filobasidium was negatively correlated with bisdemethoxycurcumin and vanilllin (Table S13). Filobasidium was more abundant in the epiphytic fungal community than in the endophytic fungal community, whereas Blumeria was the opposite (Table 1). The relative abundance of Neosetophoma in EpF was significantly (p < 0.05) higher than that in EpI (Table 1).

**DISCUSSION**

The present work investigated the effects of Epichloë endophytes on phyllosphere microbial communities and leaf metabolites of A. inebrians. In particular, we carried out the correlation analysis of the phyllosphere microbes and leaf metabolites and found there are many complex and close correlations between them.

---

**Table 4. Top 10 up and down differential metabolites**

| Metabolite               | Class                        | EL(Normalized abundance) | EF(Normalized abundance) | Log 2FC (Fold change) | Up/Down |
|--------------------------|------------------------------|---------------------------|---------------------------|-----------------------|---------|
| Methylergonovine         | Alkaloids                    | 2.32 x 10^{-3}            | 1.36 x 10^{-4}            | 4.09                  | Up      |
| Enterodiol               | Phytoestrogens               | 1.08 x 10^{-3}            | 7.34 x 10^{-5}            | 3.87                  | Up      |
| 3-Indoleacetonitrile     | Indole Derivatives           | 2.45 x 10^{-4}            | 2.36 x 10^{-5}            | 3.37                  | Up      |
| Viloxazine               | Ethers                       | 8.31 x 10^{-3}            | 8.82 x 10^{-4}            | 3.23                  | Up      |
| Suberic acid             | Lipids-Fatty Acids           | 3.66 x 10^{-4}            | 4.4 x 10^{-5}             | 3.06                  | Up      |
| Pentosidine              | Carbohydrates and Derivatives| 7.49 x 10^{-4}            | 9.58 x 10^{-5}            | 2.97                  | Up      |
| beta-Octylglucoside      | Carbohydrates and Derivatives| 2.61 x 10^{-5}            | 3.35 x 10^{-6}            | 2.96                  | Up      |
| p-Coumaric acid          | Organic Acids                | 2.19 x 10^{-4}            | 3.11 x 10^{-5}            | 2.81                  | Up      |
| Phenoxyazine             | Organonitrogen Compounds     | 2.95 x 10^{-4}            | 5.27 x 10^{-5}            | 2.48                  | Up      |
| 4-hydroxybenzoate        | Organic Acids                | 1.18 x 10^{-3}            | 2.18 x 10^{-4}            | 2.44                  | Up      |
| trans-Ferulic acid       | Carboxylic Acid Derivatives  | 3.08 x 10^{-3}            | 1.30 x 10^{-2}            | 2.08                  | Down    |
| 9-Decen-1-ol             | Carboxylic Acid Derivatives  | 6.79 x 10^{-4}            | 3.07 x 10^{-5}            | 2.18                  | Down    |
| Vanillin                 | Phenols                      | 2.73 x 10^{-5}            | 1.54 x 10^{-4}            | 2.50                  | Down    |
| Diosmetin                | Flavones                     | 1.86 x 10^{-5}            | 1.05 x 10^{-4}            | 2.50                  | Down    |
| Zotepine                 | Organonitrogen Compounds     | 4.59 x 10^{-6}            | 4.02 x 10^{-5}            | 3.13                  | Down    |
The effect of *Epichloë* endophytes on phyllosphere microbial communities

Previous studies using high-throughput sequencing approaches showed that Proteobacteria was the most abundant phylum in phyllosphere bacterial communities (Whipps et al., 2010; Stone and Jackson, 2016). Rastogi et al. (2012) reported four bacterial phyla on lettuce foliage; Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria. Our results demonstrated that Proteobacteria was the most prominent phylum, and the majority of the remaining OTUs of the phyllosphere bacterial community of *A. inebrians* belonged to the Firmicutes, Actinobacteria, and Bacteroidetes.

Our results partially supported the first hypothesis that the *Epichloë* endophyte as a keystone species would modulate the diversity of phyllosphere bacterial and fungal communities on *A. inebrians*. Roberts abd Lindow (2014) found that loline alkaloids produced by some *Epichloë* endophytes can be exploited by epiphytic bacteria, thus regulating the phyllosphere epiphytic bacterial community. However, Nissinen et al. (2019) concluded that the effect of *Epichloë* on the phyllosphere endophytic bacterial communities in *Schedonorus phoenix* (tall fescue) plants was negligible. In studies on the root and rhizosphere soil bacteria of *A. inebrians*, Ju et al. (2020) discovered that *Epichloë* endophyte reduced the Shannon diversity of the root-associated bacterial community. Our diversity results indicated that the *Epichloë* endophyte increased the diversity of phyllosphere (endophytic and epiphytic) bacterial communities on *A. inebrians*. These differing findings of studies on the effects of *Epichloë* endophytes on and within the same and different endophyte/host grass associations likely indicate the presence of different mechanisms.

Previous studies found that Ascomycota and Basidiomycota were the most abundant phyla in the phyllosphere fungal community (Bálint et al., 2015; Coleman-Derr et al., 2016), which is consistent with our research results. Nissinen et al. (2019) reported that *Epichloë* endophytes significantly influenced the community structures of phyllosphere endophytic fungal communities of *S. phoenix*. However, Cargo et al. (2020) found that *Epichloë* infection status had no significant effect on the phyllosphere endophytic fungi community of *Poa bonariensis*. With the underground fungi associated with *A. inebrians*, Zhong et al. (2018) showed that *Epichloë* endophyte reduced the diversity of the root-associated fungal community. Our present study showed that the *Epichloë* endophyte promoted the diversity of the phyllosphere endophytic fungi community of *A. inebrians*. *Epichloë* endophyte may modulate the phyllosphere fungal community via a variety of channels; direct interactions between fungi, such as competition (include nutrition and niche) or interspecific coexistence, or special molecular mechanisms that affect plants physiology and thus affecting the entire phyllosphere fungal community.

Phyllosphere microbial endophytic and epiphytic communities

Through isolation and culture, L. L. Wang et al. (2018b) found that the diversity of phyllosphere endophytic bacteria in tomatoes was lower than that of epiphytic bacteria. Similarly, another study also utilizing traditional culturing techniques suggested that epiphytic fungi had higher diversity and richness than endophytic fungi in *Pteroceltis tatarinowii* foliage (Chai et al., 2016). However, through high-throughput sequencing, Rezamahalleh et al. (2019) found that the population density of the phyllosphere epiphytic bacteria was lower than the endophytic community of Sugarcane. Our study also indicated that the diversity of the phyllosphere endophytic bacterial community was higher than that of epiphytic bacteria on *A. inebrians*. This difference from the finding of L. L. Wang et al. (2018b) and Chai et al. (2016) may be a consequence of the difference in sensitivity and detection abilities of traditional methods, such as isolation and microscopic techniques, and molecular biology techniques (Rastogi et al., 2012). However, other factors may be important as the study of tomato plants carried out using high-throughput sequencing concluded that the diversity of the endophytic fungal community was lower than that of epiphytic fungi (Dong et al., 2020). Ren et al. (2015) indicated that the response of phyllosphere bacterial communities to elevated CO₂ and soil temperature were affected by the endophytic and epiphytic environment of the rice phyllosphere. Our data also showed that the diversity of phyllosphere endophytic and epiphytic fungal communities were significantly different. Another likely explanation of the diverging findings on
epiphytic and endophytic communities may be that temperature, humidity, and other environmental stresses can affect epiphytic fungi colonizing on plant surfaces, whereas endophytic fungi have less exposure to environmental variability. However, endophytic fungi will have greater exposure to plant defense responses (Gomes et al., 2018).

There are some important insights from this study regarding some fungal genera and their interaction with A. inebrians leaves (Table 1). *Blumeria graminis* is the cause of powdery mildew disease of *A. inebrians* and as has been shown by pathogenicity studies (Sabzalian et al., 2012; Xia et al., 2015; Xia et al., 2016), the presence of *E. gansuensis* reduces the severity of this disease. The findings of this current study are in agreement with above studies. However, in this current study *Blumeria* was almost entirely growing endophytically, probably as hyphae that had penetrated into the epidermal cells, forming haustoria (Lambertiucci et al., 2019). The near absence in the epiphytic population likely reflects the absence of chains of conidia characteristic of this disease, a result of the prevailing low humidity conditions following the initial leaf infection via airborne conidia. This reflects the apparent absence of disease symptoms on the leaves that were collected for use in the study. Presumably chains of conidia would develop on leaves under high-humidity conditions.

Two other genera of special interest are *Mycosphaerella* and *Phaeosphaeria*. These two teleomorphic genera contain many anamorphic genera, some which are pathogens of grasses, including *Septoria* and *Stagonospora* (Cunfer, 1999; Crous et al., 2009). Their presence both endophytically and epiphytically in apparently disease-free EI and EF leaves probably indicates that they are present as incipient point infections. Also detected in this study were *Filobasidium* and *Symmetrospora*. These genera, belonging to Basidiomycota, contain yeasts including red yeasts. Of particular interest is that *Filobasidium* was present almost entirely as an epiphyte in both EI and EF leaves, whereas Symmetrospora was present both epiphytically and endophytically in both EI and EF plants. It is interesting to speculate how this yeast species invades leaves and what are the consequences of its presence. Another genus of interest is *Mortierella*, a zygomycete genus. This was present in both EI and EF leaves, almost entirely growing endophytically.

The effect of *Epichloë* endophytes on leaf metabolites

Our present study found that the infection of *Epichloë* endophytes altered the class and content of leaf metabolites of *A. inebrians*, which fully supported our second hypothesis. At present, metabolomics plays an important role in understanding plant physiology and stress resistance (Bowne et al., 2012; Chen et al., 2013). Previous studies about the effects of *Epichloë* endophytes on host plant metabolites mainly focused on alkaloids (Franzluebbers and Hill, 2005), root exudates (Guo et al., 2015), and volatile organic compounds (Rostás et al., 2015). Alkaloids are the main differences in metabolites between EI and EF grasses; the endophyte-grass symbionts can produce four main kinds of important alkaloids, including indole-diterpene, pyrrolopyrazine, ergot alkaloids, and pyrrolizidine, which are not detected in endophyte-free plants (Schardl et al., 2013; Young et al., 2015). In 1984, ergovine and ergonovinine were isolated from *A. inebrians* for the first time (Zhang and Zhu, 1984). Later, ergonovine and erginine were detected and identified as the most important alkaloids in the *A. inebrians* plants host to an *Epichloë* species (Miles et al., 1996). Some studies further detected the class, impact factors and the cytotoxic effects of ergot alkaloid in *Epichloë* endophyte-infected *A. inebrians* plants (Zhang et al., 2011a; 2011b, 2012, 2014). Moreover, Song et al. (2020) suggested that in addition to toxic alkaloids, other metabolites isolated from the *Epichloë* endophytes symbionts had significant antifungal, anti-insect, and phytotoxicity activities. Guo et al. (2015) reported that endophyte status influenced root exudate composition of tall fescue, including phenolic and organic carbon content. Rostás et al. (2015) demonstrated that colonization by an *Epichloë* reduced the total amount of root volatile organic compounds in *Festuca pratensis* × *Lolium perenne*. Our present study detected various differential metabolites and found that the *Epichloë* endophyte importantly affected purine derivatives, indole derivatives, phenols, and flavonoids in *A. inebrians* leaves. Although the biological function of the alkaloids extracted from the leaves of *A. inebrians* with *Epichloë* endophytes is very important, the non-alkaloid metabolites should not be ignored either.

The impacting factors on and of phyllosphere microbes

Many previous studies shown that biotic factors, such as host species and genotypes (Bodenhausen et al., 2013), and abiotic factors such as geographical location (Rastogi et al., 2012) and climate...
(Kim et al., 2012) can impact plant phyllosphere microbial communities. In addition, the morphological and chemical properties (such as leaf thickness, nitrogen, and phosphorus content) (Yadav et al., 2005; Hunter et al., 2010), secondary metabolites (Ruppel et al., 2008), and volatile organic compounds (Gao et al., 2005) of plant leaves also play important roles in the composition of the phyllosphere microbial community. Ruppel et al. (2008) reported that the phyllosphere bacterial population densities of four plants (Brassica juncea, Brassica campestris, Cichorium endivia, and Spinacia oleracea) were positively correlated with β-carotene and negatively correlated with 2-phenylethyl glucosinolates. Yadav et al. (2005) found that the population size of phyllosphere epiphytic bacteria was negatively correlated with total phenolics content of leaves of eight Mediterranean perennial species. This is consistent with our result that phenols such as vanillin and coniferol were negatively correlated with Actinobacteria, Epsilonbacteraeota, Firmicutes, and Fusobacteria of the phyllosphere bacterial community. At present, the studies on the effect of plant metabolites on plant-associated microbes were mainly concentrated belowground. The rhizosphere soil metabolites have been reported to impact rhizosphere microbial communities (Massalha et al., 2017; Yuan et al., 2018); for example, the metabolites of tomato rhizosphere soil were significantly positively correlated with the bacterial phylogeny (Wen et al., 2020). Moreover, the effect of Epichloë endophytes on belowground microorganisms may be through secondary metabolites (Vandegrift et al., 2015; Rojas et al., 2016; Soto Barajas et al., 2016). On the other hand, plant-associated microbes play an important role in the production and utilization of plant secondary metabolites (Wei et al., 2017) and phytohormones (Lebeis et al., 2015). Therefore, there are complex and close relationships between plant metabolites and plant-associated microbes. They interact with each other and jointly act on the growth and development of plants.

Limitations of the study
This study has provided fresh insights into the bacterial and fungal communities of mature, apparently disease-free leaves of the grass species A. inebrians that is growing in the field in the summer under hot dry conditions. In addition, this study looked at the impact on the systemic symptomless mutualistic Epichloë fungal endophyte on the bacterial and fungal communities and the production of secondary metabolites. Our key findings included that the diversity of the phyllosphere bacterial and fungal communities in leaves of EI plants was higher than that in leaves of EF plants, and the presence of the Epichloë endophyte altered the classes and content of leaf metabolites associated with the leaves. In addition, we concluded that the endophytic and epiphytic microbial communities were significantly different. Some bacteria and fungi were present as part of the epiphytic and endophytic communities, whereas some were only present or were largely confined to the leaf surface or within the leaf. Through analysis, we found that there were multiple significant correlations between phyllosphere microbes, including the presence or absence of the Epichloë endophyte, and leaf metabolites. We did not study the leaves of EI and EF plants throughout the growing season, using a range of techniques including microscopic observation of excised leaves placed under high-humidity, noting the development of diseases and the associated pathogens. Further study will be needed to clarify the mechanism and the impacts of the Epichloë endophyte on bacterial and fungal colonization of leaves, including plant pathogens.

STAR★METHODS
Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
  - Site description and the origin of seeds
  - Sample collection
  - DNA extraction, amplification, and sequencing
  - Sequencing data and analyses of diversity
  - Metabolites extraction and LC-MS/MS analysis
- QUANTIFICATION AND STATISTICAL ANALYSIS
SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104144.

ACKNOWLEDGMENTS
We wish to thank the editor and anonymous reviewers for their valuable comments. This work was financially supported, the National Nature Science Foundation of China (31772665 and 32061123004), National Basic Research Program of China (2014CB138702), and the Fundamental Research Funds for the Central Universities (lzujbky-2020-cd04), Lanzhou University. We thank Peng He for his help in data analysis and visualization during the preparation of this manuscript.

AUTHOR CONTRIBUTIONS
Conceptualization, Z.X.X. and L.B.W.; Methodology, L.B.W., X.C., and Z.R.; Formal Analysis, L.B.W, J.Y.W., and Z.R.; Visualization, L.B.W.; Writing–Original Draft, L.B.W. and Z.X.X.; Writing–Review & Editing, M.J.C. and L.B.W.; Funding Acquisition, N.Z.B. and Z.X.X.; Resources, N.Z.B. and Z.X.X.

DECLARATION OF INTERESTS
The authors declare no competing interests.

REFERENCES
Agati, G., Azzarello, E., Pollastri, S., and Tattini, M. (2012). Flavonoids as antioxidants in plants: location and functional significance. Plant Sci. 199, 67–76.
Alcázar, R., Cuevas, J.C., Planas, J., Zarza, X., and Altabella, T. (2011). Integration of polyamines in the cold acclimation response. Plant Sci. 180, 31–38.
Andrews, J.H., and Harris, R.F. (2003). The ecology and biogeography of microorganisms on plant surfaces. Annu. Rev. Phytopathol. 38, 145–180.
Arnold, A.E., Henk, D.A., Eells, R.L., Lutzoni, F., and Vilgalys, R. (2007). Diversity and phylogenetic affinities of foliar fungal endophytes in loblolly pine inferred by culturing and environmental PCR. Mycologia 99, 185–206.
Arnold, A.E., Mejia, L.C., Kyilo, D., Rojas, E.I., Maynard, Z., Robbins, N., and Herre, E.A. (2003). Fungal endophytes limit pathogen damage in a tropical plant. Proc. Natl. Acad. Sci. U S A. 100, 15649–15654.
Bálint, M., Bartha, L., O’Hara, R.B., Olson, M.S., Otte, J., Pfenninger, M., Robertson, A.L., Tiffin, P., and Schmitt, I. (2015). Relocation, high-latitude warming and host genetic identity shape the foliar fungal microbiome of poplars. Mol. Ecol. 24, 235–248.
Bastias, D.A., Martinez-Ghersa, M.A., Ballaré, C.L., and Gundel, P.E. (2017). Epichloë fungal endophytes and plant defenses: not just alkaloids. Trends Plant Sci. 22, 939–948.
Bell-Dereske, L., Takaci-Vesbach, C., Kvlin, S.N., Emery, S.M., and Rudgers, J.A. (2017). Leaf endophytic fungus interacts with precipitation to alter belowground microbial communities in primary successional dunes. FEMS Microbiol. Ecol. 93, fxx036.
Bodenhausen, N., Horton, M.W., and Bergelson, J. (2013). Bacterial communities associated with the leaves and the roots of Arabidopsis thaliana. PLoS One 8, 118–125.
Bowne, J.B., Erwin, T.A., Juttner, J., Schnurbusch, T., Langridgep, B., Basic, A., and Roessnerae, U. (2012). Drought responses of leaf tissues from wheat cultivars of differing drought tolerance at the metabolite level. Mol. Plant 5, 418–429.
Cao, H., Ji, Y.L., Li, S.C., Lu, L., Tian, M., Yang, W., and Li, H. (2019). Extensive metabolic profiles of leaves and stems from the medicinal plant Dendrobium officinale kimura et migo. Metabolites 9, 215.
Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335–336.
Cardinale, M., Bruzetti, L., Quattrini, P., Borin, S., Puglia, A.M., Rizzi, A., Zanardini, E., Sorlini, C., Corselli, C., and Daffonchio, D. (2004). Comparison of different primer sets for use in automated ribosomal intergenic spacer analysis of complex bacterial communities. Appl. Environ Microbiol. 70, 6147–6156.
Cargo, P.D.M., Jannone, L.J., Soria, M., and Novas, M.V. (2020). Diversity of foliar endophytes in a dioecious wild grass and their interaction with the systemic Epichloë. Fungal Ecol. 47, 100945.
Chai, X.Y., Chai, G.Q., Xiang, Y.Y., Zhang, W.W., and Yin, P.F. (2016). Composition and ecological distribution of endophytic and epiphytic fungi from the foliage of Pteroceltis tatarinowii. Acta. Ecol. Sin. 36, 5163–5172.
Chambers, M.C., Maclean, B., Burke, R., Amodei, D., Ruderman, D.L., Neumann, S., Gatto, L., Fischer, B., Pratt, B., Egertson, J., et al. (2012). A cross-platform toolkit for mass spectrometry and proteomics. Nat. Biotechnol. 30, 918–920.
Chen, L., Li, X.Z., Li, C.J., Swoboda, G.A., Young, C.A., Sugawara, K., Leuchtmann, A., and Schardl, C.L. (2015). Two distinct Epichloë species symbiotic with Achnatherum inebrinans, drunken horse grass. Mycologia 107, 863–873.
Chen, N., He, R.L., Chai, Q., Li, C.J., and Nan, Z.B. (2016). Transcriptomic analyses giving insights into molecular regulation mechanisms involved in cold tolerance by Epichloë endophyte in seed germination of Achnatherum inebrinans. Plant Growth Regul. 80, 367–375.
Chen, W., Gao, Y.Q., Xie, W.B., Gong, L., Lu, K., Wang, W.S., Li, Y., Liu, X.Q., Zhang, H.Y., Dong, H.X., et al. (2014). Genome-wide association analyses provide genetic and biochemical insights into natural variation in rice metabolism. Nat. Genet. 46, 714–721.
Chen, W., Gong, L., Guo, Z.L., Wang, W.S., Zhang, H.Y., Liu, X.Q., Yu, S.B., Xiong, L.Z., and Luo, J. (2013). A novel integrated method for large-scale detection, identification, and quantification of widely targeted metabolites: application in the study of rice metabolomics. Mol. Plant 6, 1769–1780.
Christensen, M.J. (1996). Antifungal activity in grasses infected with Acremonium and Epichloë.
endophytes Australasian. Plant Pathol. 25, 186–191.

Christensen, M.J., Bennett, R.J., Ansari, H.A., Koga, H., Johnson, R.D., Bryan, G.T., Briyana, G.T., Simpson, W.R., Koolaarda, J.P., Nickless, E.M., et al. (2008). Epichloë endophytes grow by intercalary hyphal extension in elongating grass leaves. Fungal Genet. Biol. 45, 84–93.

Coleman-Derr, D., Desgarennes, D., Fonseca-Garcia, C., Gross, S., Clingenpeel, S., Wayke, T., North, G.; Visel, A.; Partida-Martinez, L.P., and Tringe, S.G. (2016). Plant compartment and biogeography affect microbiome composition in cultivated and native Agave species. New Phytol. 209, 798–811.

Crous, P.W., Summerell, B., Canegea, A., Wingfield, M.J., Hunter, G.C., Burgess, T.I., Crous, P.W., Summerell, B., Carnegie, A., et al. (2013). Species and genus? Persoonia 80, 209–239.

Dorn-Inab, S., Bassitta, R., Schwaiger, K., Bauera, D., and Franzluebbers, A.J., and Hill, N.S. (2005). Soil carbon, nitrogen, and ergot alkaloids with short-term exposure to endophyte-infected and endophyte-free tall fescue. Soil Sci. Soc. Am. J. 69, 404–412.

Franzluebbers, A.J., and Hill, N.S. (2005). Soil carbon, nitrogen, and ergot alkaloids with short- and long-term exposure to endophyte-infected and endophyte-free tall fescue. Soil Sci. Soc. Am. J. 69, 404–412.

Franzosa, E.A., Sirotta-Madi, A., Avila-Pacheco, J., Fornelos, N., Hauser, H.J., Reinker, S., Vatonen, T., Hall, A.B., Mallick, H., Molaverdi, L., et al. (2019). Gut microbiome structure and metabolic activity in inflammatory bowel disease. Nat. Microbiol. 4, 293–305.

Friesen, M.L., Porter, S.S., Stark, S.C., Wettberg, E.V., Sachs, J.L., and Martinez-Romero, E. (2011). Microbialy mediated plant functional traits. Annu. Rev. Ecol. Evol. Syst. 42, 23–46.

Gao, Y., Jin, Y.J., Li, H.D., and Chen, H.J. (2005). Volatile organic compounds and their roles in bacteriostasis in five conifer species. J. Integr. Plant Biol. 47, 499–507.

Ginestet, C. (2011). ggplot2: elegant graphics for data analysis. J. R. Stat. Soc. A. Stat. 174, 245–246.

Gomes, T., Pereira, J.A., Benhadi, J., Lino-Neto, T., and Baptista, P. (2018). Endophytic and epiphytic phyllosphere fungal communities are shaped by different environmental factors in a mediterranean ecosystem. Microb. Ecol. 76, 668–679.

Gourion, B., Rossignol, M., and Vorholt, J.A. (2006). A proteomic study of Myxobacterium extorquens reveals a response regulator essential for epiphytic growth. Proc. Natl. Acad. Sci. U S A 103, 13186–13191.

Guerrero, M.A., Brachmann, A., Begerow, D., and Persián, D. (2018). Transient leaf endophytes are the most active fungi in 1-year-old beech leaf litter. Fungal Divers. 89, 237–251.

Guo, J.Q., McCulley, R.L., and McNair, D.H.J. (2015). Tall fescue cultivar and fungal endophyte combinations influence plant growth and root exudate composition. Front. Plant Sci. 6, 183.

Hunter, P.J., Hand, P., Pink, D., Whipp, J.M., and Bending, G.D. (2010). Both leaf properties and microbe-microbe interactions influence within-species variation in bacterial population diversity and structure in the lettuce (Lactuca species) phyllosphere. Appl. Environ. Microbiol. 76, 8117–8125.

Innerebner, G., Knief, C., and Vorholt, J.A. (2011). Protection of Arabidopsis thaliana against leaf-pathogenic Pseudomonas syringae by Sphingomonas strains in a controlled model system. Appl. Environ. Microbiol. 77, 3202–3210.

Jin, J.J., Zhang, H., Zhang, J.F., Liu, P.P., Chen, X., Li, Z.F., Xu, Y.L., Li, P., and Cao, P.J. (2017). Integrated transcriptomics and metabolomics analysis to characterize cold stress responses in Nicotiana tabacum. BMC Genomics 18, 496.

Johnson, L.J., de Bonth, A.C.M., Briggs, L.R., Caradus, J.R., Finch, S.C., Fleetwood, D.J., Fletcher, L.R., Hume, D.E., Johnson, R.D., Popay, A.J., et al. (2013). The exploitation of epichloë endophytes for agricultural benefit. Fungal Divers. 60, 171–188.

Ju, Y.W., Zhong, R., Christensen, M.J., and Zhang, X.X. (2020). Effects of Epichloë gansuensis endophyte on the root and rhizosphere soil bacteria of Achnatherum inebrians under different moisture conditions. Front. Microbiol. 11, 747.

Kim, M., Singh, D., Lai-Hoe, A., Go, R., Raha, A.R., Anuindi, A.N., Chun, J.S., and Adams, J.M. (2012). Distinctive phyllosphere bacterial communities in Fusca cultivar and fungal endophyte-infected grass. Front. Microbiol. 3, 117.

Kulda, G., and Bacon, C. (2008). Clavicipitaceae endophytes: their ability to enhance resistance of Pseudomonas syringae pathogenic. Plant J. 57–71.

Köhlau, U., Larsson, K.H., Abarenkov, K., Nilsson, R.H., Alexander, J.J., Eberhardt, U., Erland, S., Haiden, K., Kjäller, R., Larsson, E., et al. (2005). UNITE: a database providing web-based methods for the molecular identification of ectomycorrhizal fungi. New Phytol. 166, 1063–1068.

Lambertiucci, S., Orman, K., Mary., Gupta, S.D., T., and Baptista, P. (2018). Endophytic and extrahaustorial proteomes of septoria and stagonospora species on small-grain cereals. Annu. Rev. Phytopathol. 51, 427–428.

Liang, Y., Wang, H.C., Li, C.J., Nan, Z.B., and Li, F.D. (2017). Effects of feeding drunken horse grass infected with Epichloë gansuensis endophyte on animal performance, clinical symptoms and physiological parameters in sheep. BMC Vet. Res. 13, 223.

Liu, R.X., Hong, J., Xu, X.O., Feng, Q., Zhang, D.Y., Gu, Y.Y., Shi, J., Zhao, S.Q., Liu, W., Wang, X.K., et al. (2017). Gut microbiome and serum metabolome alterations in obesity and after weight-loss intervention. Nat. Med. 23, 859–868.

Ma, X.S., Xia, H., Liu, Y.H., Wei, H.B., Zheng, X.G., Song, C.Z., Chen, L., Liu, H.Y., and Lou, L.J. (2016). Transcriptomic and metabolomic studies disclose key metabolism pathways contributing to well-maintained photosynthesis under the drought and the consequent drought-tolerance in rice. Front. Plant Sci. 7, 1886.

Maria, F.F., Mabel, N.C., Maria de las, M.E., and Verónica, N.I. (2020). Epichloë endophyte modifies the foliar anatomy of Lolium multiformum Lam. Symbiosis 81, 313–319.

Massalha, H., Korenblum, E., Toller, D., and Aharoni, A. (2017). Small molecules below-ground: the role of specialized metabolites in the rhizosphere. Plant J. 90, 788–807.

Matsuda, F., Okazaki, Y., Oikawa, A., Kusano, M., Nakabayashi, N., Kikuchi, J., Yonemaru, J., Kaworu, E., Masahiro, Y., and Kikuzo, S. (2012). Dissection of genotype-phenotype associations in rice grains using metabolome quantitative trait loci analysis. Plant J. 70, 624–638.

Mechaber, W.L., Marshall, D.B., Mechaber, R.A., Jobe, R.T., and Chew, F.S. (1999). Mapping leaf surface landscapes. Proc. Natl. Acad. Sci. U S A 96, 4600–4603.

Melotto, M., Underwood, W., Koczkan, Z., Nomura, K., and HE, S.Y. (2006). Plant stomata function in innate immunity against bacterial infection. Cell 126, 969–980.

Mercier, J., and Lindow, S.E. (2000). Role of leaf surface sugars in colonization of plants by
bacterial endophytes. Appl. Environ. Microb. 66, 369–374.

Miles, C.O., Lane, G.A., di Menna, M.E., Garthwaite, I., Piper, E.L., Ball, O.J.P., Latch, G.C.M., Allen, J.M., Hunt, M.B., Bush, L.P., et al. (1996). High levels of ergonovine and lysergic acid amide in toxic Achnatherum inebrians accompany infection by an Acremonium-like endophytic fungus. J. Agr. Food Chem. 44, 1285–1290.

Müller, C.B., and Krauss, J. (2005). Symbiosis between grasses and sexual fungal endophytes. Curr. Opin. Plant Biol. 8, 450–456.

Nam, K.H., Shin, H.J., Pack, I.S., Park, J.H., Kim, H.B., and Kim, C.G. (2016). Metabolic changes in grains of well-watered and drought-stressed transgenic rice. J. Sci. Food Agr. 96, 807–814.

Nissen, R., Helander, M., Kumar, M., and Saikkonen, K. (2019). Heritable Epichloë symbiosis shapes fungal but not bacterial communities of plant leaves. Sci. Rep.-UK. 9, 5253.

Oberhofer, M., Güsewell, S., and Leuchtmann, A. (2014). Effects of natural hybrid and non-hybrid Epichloë endophytes on the response of Hordeum vulgare to drought stress. New Phytol. 201, 242–253.

Partida-Martínez, L.P., and Heil, M. (2011). The microbe-free plant: fact or artifact? Front. Plant Sci. 2, 100.

Pefuelas, J., and Terradas, J. (2014). The foliar microbiome. Trends Plant Sci. 19, 278–280.

Purahong, W., and Hyde, K.D. (2011). Effects of fungal endophytes on grass and non-grass litter decomposition rates. Fungal Divers 47, 1–7.

Quast, C., Pruess, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., and Gloeckner, F.O. (2013). The SILVA ribosomal RNA gene web-based tools. Nucl. Acids Res. 41, 38–39.

Roberts, E.L., and Ferraro, A. (2015). Rhizosphere microbiome selection by Epichloë endophytes of Festuca arundinacea. Plant Soil 396, 229–239.

Rojas, X., Guo, J.O., Leff, J.W., McNear, D.H., Fierer, N., and McCulley, R.L. (2016). Infection with a shoot-specific fungal endophyte (Epichloë) alters tall fescue soil microbial communities. Microb. Ecol. 72, 197–206.

Rostás, M., Cripps, M.G., and Silcock, P. (2015). Aboveground endophyte affects root volatile emission and host plant selection of a belowground insect. Oecologia 177, 487–497.

Ruppel, S., Krumbein, A., and Schreiner, M. (2008). Composition of the phyllospheric microbial populations on vegetable plants with different glucosinolate and carotenoid compositions. Microb. Ecol. 56, 364–376.

Sabzalian, M.R., Mirlohi, A., and Sharifnab, B. (2012). Reaction to powdery mildew fungus, Blumeria graminis in endophyte-infected and endophyte-free tall and meadow fescues. Australas. Plant Pathol. 41, 565–572.

Santamaria, J., and Bayman, P. (2005). Fungal endophytes and endophytes of coffee leaves (Coffea arabica). Microb. Ecol. 50, 1–8.

Scandiani, M.M., Luque, A.G., Razori, M.V., Lucila, C.C., Takayuki, A., Kery, O., Cerovigni, G.D.L., and Spampinato, C.P. (2015). Metabolic profiles of soybean roots during early stages of Fusarium tumicainae infection. J. Exp. Bot. 66, 391–402.

Scharl, C.L., Leuchtmann, A., and Spiering, M.J. (2005). Symbioses of grasses with seedborne fungal endophytes. Annu. Rev. Plant Biol. 55, 315–340.

Scharl, C.L., Young, C.A., Hesse, U., Ammote, S.G., Andreeva, K., Calie, P.J., Fleetwood, D.J., Haws, D.C., Moore, N., Oeser, B., et al. (2013). Plant-symbiotic fungi as chemical engineers: multi-genome analysis of the clavicipitateae reveals dynamics of alkaloid loci. PLoS Genet. 9, e1003323.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Holsbe, E.B., Lesniewski, R.A., Oakley, B.L., Parks, D.H., Robinson, C.J., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microb. 75, 7575–7576.

Schmidt, M., Unterer, S., Suchodolski, J.S., Honneffer, J.B., Guard, B.C., Lidbury, J.A., Steiner, J.M., Fritz, J., and Köllé, P. (2018). The fecal microbiome and metabolome differs between dogs fed Bones and Raw Food (BARF) diets and dogs fed commercial diets. PLoS One 13, e0201279.

Song, Q.Y., Li, F., Nan, Z.B., Coulter, J.A., and Wei, W.J. (2020). Do Epichloë endophytes and their grass symbiosis only produce toxic alkaloids to insects and livestock? J. Agr. Food Chem. 68, 1169–1188.

Soto-Barajas, M.C., Zabalgozoezaoz, I., Gómez-Fuertes, J., González-Blanco, V., and Vázquez-de Aldana, B.R. (2016). Epichloë endophytes affect the nutrient and fiber content of Lollum perennne, regardless of plant genotype. Plant Soil 405, 265–277.

Stone, B.W.G., and Jackson, C.R. (2016). Biogeographic patterns between bacterial phyllosphere communities of the southern magnolia (Magnolia grandiflora) in a small forest. Microb. Ecol. 71, 954–961.
Young, C.A., Schardl, C.L., Panaccione, D.G., Flores, S., Takach, J.E., Nikki, D., Moore, N., Webb, J.S., and Jaromczyk, J. (2015). Genetics, genomics and evolution of ergot alkaloid diversity. Toxins 7, 1273–1302.

Yuan, J., Zhao, J., Wen, T., Zhao, M.L., Li, R., Goossens, P., Huang, Q.W., Bai, Y., Vivanco, J.M., Kowalchuk, G.A., et al. (2018). Root exudates drive the soil-borne legacy of aboveground pathogen infection. Microbiome 6, 156.

Zhang, X.X., Li, C.J., and Nan, Z.B. (2010). Effects of cadmium stress on growth and anti-oxidative systems in Achnatherum inebrrians symbiotic with Neotyphodium gansuense. J. Hazard. Mater. 175, 703–709.

Zhang, X.X., Li, C.J., and Nan, Z.B. (2011a). Effects of cutting frequency and height on alkaloid production in endophyte-infected drunken horse grass (Achnatherum inebrrians). Sci. China Life Sci. 54, 567–571.

Zhang, X.X., Li, C.J., and Nan, Z.B. (2011b). Effects of salt and drought stress on alkaloid production in endophyte-infected drunken horse grass (Achnatherum inebrrians). Biochem. Syst. Ecol. 39, 471–476.

Zhang, X.X., Li, C.J., Nan, Z.B., and Matthew, C. (2012). Neotyphodium endophyte increases Achnatherum inebrrians (drunken horse grass) resistance to herbivores and seed predators. Weed Res. 52, 70–78.

Zhang, X.X., Nan, Z.B., Li, C.J., and Gao, K. (2014). Cytotoxic effect of ergot alkaloids in Achnatherum inebrrians infected by the Neotyphodium gansuense endophyte. J. Agr. Food Chem. 62, 7419–7422.

Zhang, Y.J., and Zhu, Z.Q. (1984). Studies on the chemical compositions of Achnatherum inebrrians. Chem. J. Chinese Univ. 3, 150–152.

Zhong, R., Xia, C., Ju, Y.W., Li, N.N., Zhang, X.X., Nan, Z.B., Li, C.J., and Christensen, M.J. (2018). Effects of Epichloë gansuensis on root-associated fungal communities of Achnatherum inebrrians under different growth conditions. Fungal Ecol. 31, 29–36.

Zhong, R., Xia, C., Ju, Y.W., Zhang, X.X., Nan, Z.B., Li, C.J., and Christensen, M.J. (2021). A foliar Epichloë endophyte and soil moisture modified belowground arbuscular mycorrhizal fungal biodiversity associated with Achnatherum inebrrians. Plant Soil 458, 105–122.
## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Chemicals, peptides, and recombinant proteins** | | |
| GC Enhancer | TransGen Biotechnology | Cat# AG101-01 |
| KOD FX Neo (TOYOBO) | Bellink Biotechnology | Cat# KFX-201S |
| Phusion HF MM | Bellink Biotechnology | Cat# M0544L |
| PowerPlant DNA Isolation Kit | MO BIO Laboratories | Cat# 13200-100 |
| Q5® High-Fidelity DNA Polymerase | New England Biolabs | Cat# M04915 |
| Quant-iT™ dsDNA HS | Thermo Fisher Scientific | Cat# Q33120 |
| VAHTS® DNA Clean Beads | Vazyme | Cat# N411-03 |

| Deposited data | | |
|---|---|---|
| Raw data of bacteria | This paper | NCBI: PRJNA795658 |
| Raw data of fungi | This paper | NCBI: PRJNA795665 |

| Oligonucleotides | | |
|---|---|---|
| 335F | Tiagen Biochemical Technology (Beijing) Co., LTD | 5’-CADACTCCTACGGGAGGC-3’ |
| 769R | Tiagen Biochemical Technology (Beijing) Co., LTD | 5’-ATCCTGTGGTGMCCVMCR-3’ |
| ITS1F | Tiagen Biochemical Technology (Beijing) Co., LTD | 5’-CCCTTGTCATTAGAGGAAAT-3’ |
| ITS2R | Tiagen Biochemical Technology (Beijing) Co., LTD | 5’-GCTGCCGTTTTCATCGATGC-3’ |

| Software and algorithms | | |
|---|---|---|
| Analyst TF v1.7 | AB Sciex | RRID:SCR_015785 |
| ggplot2 v3.2.1 | Ginenet, 2011 | https://cloud.r-project.org/web/packages/ggplot2/index.html |
| Mothur v1.42.0 | Schloss et al., 2009 | RRID:SCR_011947 |
| Origin 2021 | OriginLab | https://www.originlab.com/ |
| ProteoWizard | Chambers et al., 2012 | RRID:SCR_012056 |
| QIIME v1.7.0 | Caporaso et al., 2010 | RRID:SCR_008249 |
| R v4.0.2 | R | RRID:SCR_001905 |
| SigmaPlot v12.5 | Systat Software | http://www.systatsoftware.cn/download-sigmaPlot.html |
| Silva v138.1 | Quast et al., 2013 | https://www.arb-silva.de/ |
| SPSS Statistics v26.0 | IBM | RRID:SCR_019096 |
| TripleTOF 5600 | AB Sciex | https://sciex.com.cn/products/mass-spectrometers/qtof-systems/tripletof-systems/tripletof-5600-system |
| UNITE v7.2 | Köjalg et al., 2005 | RRID:SCR_006518 |
| Usearch v8.0.1623 | Edgar, 2013 | http://www.drive5.com/usearch/ |
| XCMS v3.7.1 | Want et al., 2006 | https://bioconductor.org/packages/release/bioc/html/xcms.html |
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and materials should be directed to and will be fulfilled by the lead contact, Xingxu Zhang (xxzhang@lzu.edu.cn).

Materials availability
This study did not generate new unique reagents.

Data and code availability
The sequence data used in this study are available at NCBI database (accession numbers are listed in Key resources table). The matrix of OTUs by sample used to generate the plots in this study are provided in Tables S16 and S17.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS
Our study does not use experimental models typical in the life sciences.

METHOD DETAILS

Site description and the origin of seeds
This study site was situated at the College of Pastoral Agriculture Science and Technology, Yuzhong campus (104°39'E, 35°89'N, and altitude 1653 m) of Lanzhou University. A total of 12 plots (each 4.8 X 4.0 m) were established in 2017 in this experimental field. Each plot was divided into two sub-plots by a 1 m deep concrete wall. One sub-plot of each plot was planted with endophyte-infected (EI) plants, and the other sub-plot was planted with endophyte-free (EF) plants.

The plants used in this study originated from a single A. inebrians plant in which 20 tillers that had been shown to be infected by E. gansuensis by microscopic examination of leaf sheathes stained by aniline blue (Li et al., 2004). The presence of seldom-branched intercellular hyphae with non-staining septa confirmed that tillers were infected by the Epichloë endophyte. In 2011, seeds were collected from this plant and were divided into two parts: one treated with thiophanate-methyl to eliminate the ability of endophytes to infect seedlings, and the other was not fungicide treated. To obtain stocks of EI and EF seeds for experimental use, in 2012, 200 seedlings from each of the two parts of this single plant seed collection were planted separately at the experimental site. Representative plants of these two populations were examined to confirm that their endophyte status was correct (Xia et al., 2015, 2016, 2018; Ju et al., 2020; Zhong et al., 2018, 2021).

Sample collection
Leaf samples were collected from field growing plants in July 2019. Samples from eight plots were randomly selected, EI and EF samples were obtained from two sub-plots of each plot. The method of five points sampling was used to obtain the leaves of A. inebrians plants. The 3rd or 4th newest leaf of a tiller was selected from the plants in five sampling sites of each sub-plot and mixed to form mixed leaves samples. Fifteen - 17 cm long leaf segments were cut with sterilized scissors, without sheath and tip. These samples were brought back to the laboratory in liquid nitrogen containers, and stored −80°C refrigerator until DNA and metabolites extraction. Each individual leaf sample was divided into two parts, one for microbiome sequencing and the other for metabolite detection.

DNA extraction, amplification, and sequencing
A total of 16 leaf samples (eight samples of EI, eight samples of EF) were used for the detection of endophytic and epiphytic microbes. Epiphytic microbes were washed from leaf surfaces. Leaves (5 g) were transferred into 50 mL plastic tubes filled with 30–40 mL PBS buffer, along with two blank controls without adding leaf sample, followed by oscillation for 30–60 min at 150–200 r/min, sonication for 5 min, and further oscillation for 30–60 min at 150–200 r/min. The leaves were removed and the suspension was
centrifuged at 10,000 g for 10 min to obtain precipitates containing bacteria, fungal spores and hyphae dislodged from the surface of leaves. For endophytic microbes, the above harvested leaves were surface-sterilized in 75% ethanol for 1 min, 1% sodium hypochlorite for 2 min and 75% ethanol for 30 s, and then washed three times in sterile water. Subsequently, the treated leaves were ground and homogenized with liquid nitrogen.

The DNA was extracted using the PowerPlant DNA Isolation Kit (MO BIO Laboratories) according to the manufacturer’s protocol. Two blank controls were set up with the same volume of ddH2O instead of the sample. The V3-V4 region of the bacterial 16S rRNA gene was amplified with the primer pair (338F: 5'-CA DACTCTACGGGAG GC-3' and 769R: 5'-GCTGCGTTCTTCATCGATGC-3') (Dorn-Inab et al., 2015). The fungal internal transcribed spacer 1 (ITS1) region of rRNA gene was amplified with primers pair (ITS1F: 5'-CTTGTCATTAGAGGAAATC-3' and ITS2R: 5'-GCTGCTTCTTATCGATGC-3') (Cardinale et al., 2004). PCR amplification was performed in a total volume of 50 μL, which contained 10 μL Buffer, 0.2 μL Q5 High-Fidelity DNA Polymerase, 1 μL dNTP, 10 μM of each primer and 60 ng genomic DNA. For each amplification, two negative controls were set up with the same volume of ddH2O instead of DNA template. Thermal cycling conditions were as follows: an initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 30 s, 50°C for 30 s and 72°C for 30 s, with a final extension at 72°C for 7 min. The PCR products from the first step PCR were purified through VAHTSTM DNA Clean Beads. A second round PCR was then performed in a 40 μL reaction which contained 20 μL 2× Phusion HF MM, 8 μL ddH2O, 10 μM of each primer and 10 μL PCR products from the first step. Thermal cycling conditions were as follows: an initial denaturation at 98°C for 30 s, followed by 10 cycles at 98°C for 10 s, 65°C for 30 s and 72°C for 30 s, with final extension at 72°C for 5 min. Finally, all PCR products were quantified by Quant-iT dsDNA HS Reagent and pooled together. High-throughput sequencing analysis of bacterial and fungal rRNA genes was performed on the purified, pooled sample using the Illumina novaseq6000 at Biomarker Technologies Corporation (BMK), Beijing, China.

**Sequencing data and analyses of diversity**

The bacterial 16S rDNA and fungal ITS nucleotide sequences were assembled and filtered; reads with ambiguous nucleotides, a quality score of less than 15, lacking complete barcode and primer were deleted and excluded from further analysis, and then the primer region was removed. Usearch software (v.8.0.1623) was used to cluster sequences and obtain OTUs at 97% similarity level. After filtering out the OTUs whose sequence number was less than 5 / 100000 of the total sequence numbers, taxonomic annotation of OTUs was carried out based on the taxonomy database of Silva (bacteria) (v.138.1) and UNITE (fungi) (v.7.2). The abundance information of the OTUs was normalized by using the sequence number standard, which corresponded to the sample with the minimum sequence. The alpha diversity index including Shannon index (https://mothur.org/wiki/Shannon/), Simpson (https://mothur.org/wiki/Simpson/), Chao1 (https://mothur.org/wiki/Chao1/) and ACE (https://mothur.org/wiki/ACE/) were calculated using Mothur software (v.1.42.0). Non-metric multi-dimensional scaling (NMDS) was performed using QIIME (v.1.7.0) with Bray-Curtis distance calculated from the bacterial and fungal OTU community matrix.

**Metabolites extraction and LC-MS/MS analysis**

Twelve leaf samples (six samples of EI, six samples of EF) were randomly selected from the 16 microbiome sequencing samples for the detection metabolites. For the non-targeted metabolites, leaves (5 g) were slowly rinsed by washing three times with sterile water to remove soil and impurities on the leaf surface. 50 mg leaf and 400 μL extract solution (acetonitrile: methanol = 1: 1) containing isotopically-labelled internal standard mixture was added to an EP tube. After 30 s vortex, the samples were sonicated for 10 min in an ice-water bath. Then the samples were incubated at −40°C for 1 h and centrifuged at 12,000 rpm for 15 min at 4°C. 400 μL of supernatant was transferred to a fresh tube and dried in a vacuum concentrator at 37°C. Then the dried samples were dissolved in 200 μL of 50% acetonitrile by sonication on ice for 10 min. The solution was then centrifuged at 13,000 rpm for 15 min at 4°C, and 75 μL of supernatant was transferred to a sample bottle for LC/MS analysis. The quality control (QC) sample was prepared by mixing an equal aliquot of the supernatants from all of the samples.

The UHPLC separation was carried out using a ExionLC Infinity series UHPLC System (AB Sciei), equipped with a UPLC BEH Amide column (2.1 x 100 mm, 1.7 μm, Waters). The mobile phase consisted of 25 mmol/L ammonium acetate and 25 mmol/L Ammonia hydroxide in water (pH = 9.75) (A) and acetonitrile (B). The analysis was carried with an elution gradient as follows: 0–0.5 min, 95% B; 0.5–7.0 min, 95%–65% B; 7.0–8.0 min, 65%–40% B;
8.0–9.0 min, 40% B; 9.0–9.1 min, 40%–95% B; 9.1–12.0 min, 95% B. The column temperature was 25°C. The auto-sampler temperature was 4°C, and the injection volume was 2 μL (pos) or 2 μL (neg), respectively.

The TripleTOF 5600 mass spectrometry (AB Sciex) was used for its ability to acquire MS/MS spectra on an information-dependent basis (IDA) during an LC/MS experiment. In this mode, the acquisition software (Analyst TF v.1.7) continuously evaluates the full scan survey MS data as it collects and triggers the acquisition of MS/MS spectra depending on preselected criteria. In each cycle, the most intensive 12 precursor ions with intensity above 100 were chosen for MS/MS at collision energy (CE) of 30 eV. The cycle time was 0.56 s. ESI source conditions were set as following: Gas 1 as 60 psi, Gas 2 as 60 psi, Curtain Gas as 35 psi, Source Temperature as 600°C, Declustering potential as 60 V, Ion Spray Voltage Floating (ISVF) as 5000 V or −4000 V in positive or negative modes, respectively.

MS raw data (. wiff) files were converted to the mzXML format by ProteoWizard, and processed by R package XCMS (v.3.7.1). The process includes peak deconvolution, alignment and integration. Minfrac and cut off are set as 0.5 and 0.3 respectively. MS2 database of BMK was applied for metabolites identification. QC samples with samples correlation <0.7 or the relative standard deviation (RSD) >30% were removed. The data were normalized by the method of total peak area normalization, each metabolite of each sample divided by the total peak area of the sample.

QUANTIFICATION AND STATISTICAL ANALYSIS

All further analyses were performed using R software (v.4.0.2), unless otherwise specified. Visualizations were plotted using ggplot2 (v.3.2.1), Origin 2021 and SigmaPlot (v.12.5). One-way analysis of variance (ANOVA), two-way ANOVA and Fishers least significant differences (LSD) test were performed to compare differences in relative abundance at the phylum and genus level and in alpha diversity across different treatments by IBM SPSS Statistics (v.26.0). In all tests, p value < 0.05 was considered statistically significant. For correlation analysis, if the sample had both metabolite data and microbial data, the sample was used for Spearman rank correlation. Metabolite data and microbial data were paired based on individual leaf samples to avoid the influence of sample diversity on correlation results.