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The differences and overlaps in the seed-resident microbiome of four *Leguminous* and three *Gramineous* forages

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Summary

Given the important roles that seed-borne endophytes can play on their plant hosts, comprehensive studies of the bacterial and fungal communities of seeds are of great importance. In this study, we assessed the seed endophytes of three gramineous (*Avena sativa*, *Elymus sibiricus* and *Elymus dahuricus*) and four leguminous (*Vicia villosa*, *Trifolium repens*, *Trifolium pretense* and *Medicago sativa*) forages using high-throughput sequencing. In total, 1013 distinct bacterial operational taxonomic units (OTUs) and 922 fungal OTUs were detected, with bacteria and fungi per sample ranging from 240 to 425 and 261 to 463 respectively. These seven forages shared a high number of potentially beneficial taxa, including *Bacillus*, *Pantoea*, *Candida* and *Helotiales*, but the relative proportion of these taxa was different in each seed. Fungal communities were clustered more distinctively by host genotypes than bacterial. Some bacterial taxa may be involved in the recruitment of genera from the same phylum. Three *Pantoea* sp. and five *Bacillus* sp. were isolated from seeds, and all showed positive effects on *Medicago sativa* germination rate under salt stress, and of these, *Bacillus subtilis* Es-1 and *Pantoea agglomerans* Ed-3 performed best, but their influence was affected by the seed's microbiome. Rather than simply promoting host plant growth directly, some taxa may also participate in organizing the assembly of plant microbiomes which will influence seed response to biological factors. This study uses a new, high-throughput sequencing based strategy to identify beneficial strains and analyse the interactions between microorganisms and plants to maximize microbial functions in long-term agricultural practices.

Introduction

Internal plant tissues usually contain a variety of microbial communities (Hardoim *et al*., 2015). A broad range of bacteria and fungi has been isolated or sequenced from different plant compartments, including seeds (Truyens *et al*., 2015), shoots (Rojas *et al*., 2016) and leaves (Yumlembam and Borkar, 2014). Most of these microorganisms are referred to as commensals, with unknown or yet unknown functions in plants, and only 5~10% of the total endophytes confer beneficial growth effects (Hallmann *et al*., 1997). Endophyte communities have been reported to play important roles in promoting plant growth (Compant *et al*., 2009; Andreozzi *et al*., 2019) and improving plant resistance to biotic and abiotic stresses (Gond *et al*., 2015; Lanza *et al*., 2019). Plant microbiota might also play potential roles in reducing postharvest food loss (Buchholz *et al*., 2018). These 'plant probiotics' offer a safe and environmentally friendly alternative to the current dependence on chemical pesticides (Molina-Santiago and Matilla, 2019; Thomashow *et al*., 2019). Although many microorganisms that promote plant growth have been reported, they may occasionally outcompete a host's resident microbiome, which has the potential to unexpectedly affect the host (Castro-Sowinski *et al*., 2007). The structure of the *Brassica napus* seed microbiome, for example, has been shown to affect the interactions of symbionts and pathogens (Rybakova *et al*., 2017). In this era of climate change,

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maximizing microbial functions in plant growth has become a prerequisite for the sustainability of global agriculture; however, managing species-rich communities of plant-associated microbiomes remains a major challenge (Toju *et al*., 2018). While we have started to understand the complexity of the involved microorganisms and their interactions, more research is needed on plant–microbiome interactions to help maximize microbial functions in agroecosystems (Mitter *et al*., 2016).

Plant seeds are the starting point for the next generation, and diverse microorganisms have evolved alongside them (Truyens *et al*., 2015; Shahzad *et al*., 2018). Seed endophytes have been reported to assist seed preservation and germination, seedling growth and stress tolerance (Cheesanford *et al*., 2006; Goggin *et al*., 2015; Shearin *et al*., 2018; Shahzad *et al*., 2018). In plant microbial assembly, small differences in early colonization processes may lead to large community differences during later life stages (Fukami, 2015). The microbial component of healthy seeds appears to be inherited between plant generations and is likely to represent an important 'seed' for the host microbiome. Modifying the microbial composition from its starting point could play a key role in promoting plant growth and maintaining plant health. Given the important roles seed-borne endophytes have on their plant hosts, comprehensive studies of seed bacterial and fungal communities should be a research priority (Toju *et al*., 2018). However, seed microbiota studies in the past have been largely based on culture-dependent investigations (Shahzad *et al*., 2018) and have lagged behind leaf and root (Coleman-Derr *et al*., 2016; Naylor *et al*., 2017). Seeds from ecologically and geographically diverse (species) were reported to harbour characteristic microbiota (Links *et al*., 2014; Barret *et al*., 2015; Klaedtke *et al*., 2016). Comprehensive research on seed endophytes using high-throughput sequencing has only begun in recent years and was mainly focused on field-crop plants such as Maize (Yang *et al*., 2013), *Rice* (Midha *et al*., 2016), *Phaseolus vulgaris* (Klaedtke *et al*., 2016) and *Barley* (Yang *et al*., 2017). A comprehensive understanding of endophyte microbiome in forage seeds is therefore yet to be uncovered.

According to previous studies, plants that can survive in extreme environments often have a unique microbiome which plays a vital role in improving the stress resistance and productivity of its host (Coleman-Derr *et al*., 2016; Pitzschke, 2016). Microorganisms isolated from plants grown in semi-arid environments can promote the growth and improve stress tolerance of a variety of crops (Marasco, *et al*., 2012; Mengual, *et al*., 2014; Rolli, *et al*., 2014). The Qinghai–Tibet Plateau is the highest altitude plateau in the world and is often referred to as the 'Roof of the World' and the 'Third Pole'. Due to its harsh climate, pastures in the Plateau are mainly occupied by gramineous forage, while vulnerable legumes are in short supply; therefore, only a few leguminous seeds could be harvested. The balance between leguminous and gramineous forages is important for enriching forage nutrition and improving soil properties (Xiang *et al*., 2018; Du *et al*., 2019), and thus, introducing high-quality adaptable legumes to the Qinghai–Tibet Plateau is a priority. In this study, we investigated the seed-resident microbiome of four *Leguminous* and three *Gramineous* forages to address the following questions: (i) What are the main differences in the endophytes associated with these seeds and which of them may be potentially beneficial strains to these forages, especially to the Qinghai–Tibet Plateau grass? (ii) What does the interaction relationship between microorganisms look like? (iii)We examined whether the potentially beneficial strains in the seeds could be isolated and whether they promoted plant germination and growth? (iv) Can the beneficial effects of certain isolated microorganisms be affected by the seed-resident microbiomes?

Results

The global profile of the seed-resident microbiome

A total of 720 608 and 721 979 clean reads were generated from 16S rRNA gene and ITS sequencing respectively. Sequences per sample varied from 30 034 to 39 839 reads (median 34 259) for bacteria and 30 347 to 38 215 reads (median 35 007) for fungi. These reads were assigned to 1013 distinct bacterial operational taxonomic units (OTUs) and 922 fungal OTUs, ranging from 240 to 425 and 261 to 463 per sample for bacteria and fungi respectively. Of the 1013 observed bacterial OTUs, 67 were shared between all seven samples, which accounted for 74.9% in all sequence reads. Between 43 and 146 bacterial OTUs were unique to each community, but they were in low abundance (0.2% to 1.5%) (Fig. 1A). There were 115 common fungal OTUs in the seven forages, representing 95.5% of fungal sequence reads. As with the bacterial OTUs, the fungal OTUs unique to each forage were found in low abundance (Fig. 1B). In conclusion, the OTUs specific to each forage seed were found in low abundance and the OTUs common to all seven forages were found in high abundance.

Microbial community diversity in each forage seed

No significant differences were detected when the bacterial and fungal communities associated with Leguminosae and Poaceae were compared using Chao1, Simpson's and Shannon indices by independent-sample t test. The highest bacterial community diversity was

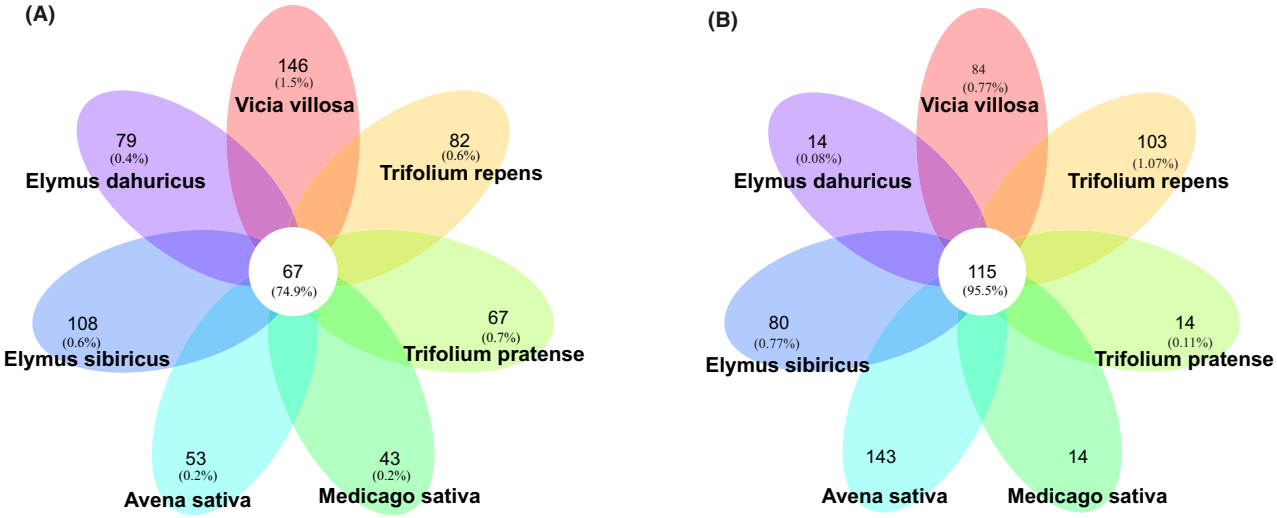

Fig. 1. Venn plot of bacterial (A) and fungal (B) communities of the seven forage seeds. Venn diagrams show absolute number of operational taxonomic units (OTUs) and the relative sequence percentage in the total number of OTUs.

seen in *Vicia villosa* seeds, followed by *Trifolium repens*. *Avena sativa* had the lowest bacterial diversity, but a high fungal diversity. *Elymus sibiricus* and *Elymus dahuricus* had lower fungal diversity than other seeds (Fig. 2A). A beta diversity analysis, based on weighted UniFrac distances (Bray–Curtis), was performed (Fig. 2B) and showed that the fungal communities of the seven seeds were more distinctively clustered by the host genetic background than bacterial (Fig. 2B; Fig. S1). The bacterial and fungal communities of *Avena sativa* were less like those found in *Elymus sibiricus* and *Elymus dahuricus*, despite all of them being gramineous, and were more like the microbiomes of leguminous seeds. In particular, the bacterial communities of *Avena sativa* were clustered together with those of *Trifolium repens* and *Medicago sativa*.

Differences in bacterial communities across forages

After normalization and taxonomic assignment, some highly abundant genera were seen in all seven forage species, although their relative abundance was highly variable. Bacterial communities were composed mainly of *Proteobacteria* (54~90%, per sample) and *Firmicutes* (5~41%). *Proteobacteria* were abundant in all forage seeds, especially in *Medicago sativa* (90%) and *Avena sativa* (86%), while *Firmicutes* were only abundant in *Vicia villosa* (25%), *Elymus sibiricus* (41%) and *Elymus dahuricus* (24%). *Proteobacteria* was represented by the classes of *GammaProteobacteria* (28~80%), *AlphaProteobacteria* (2~31%) and *BetaProteobacteria* (3~10%), and *Firmicutes* mainly represented by *Bacilli* (5~38%) (Table 1).

About 73% of the bacterial OTUs could be identified at the genus level (Fig. S2A). The circular visualization shows the relative abundance of the ten most abundant bacterial genera in the seven forage seeds (Fig. 3). The relative abundance of each genera was more balanced in *Vicia villosa* and *Trifolium repens* than in other seeds. *Vicia villosa* had a higher abundance of *Acinetobacter* (8%), *Sphingomonas* (5%) and *Lactobacillus* (6%), but had lower abundance of *Bacillus* (4%) and *Pantoea* (2%) than other seeds. *GammaProteobacteria* comprised more than half of the bacteria microbiota (63~80%) in *Trifolium pratense*, *Medicago sativa* and *Avena sativa*, due to the dominance of *Pantoea* (27%) and *Aeromonas* (11%) in *Trifolium pratense*, *Pantoea* (36%) and *Salmonella* (12%) in *Medicago sativa* and *Pantoea* (42%) in *Avena sativa*. *Elymus sibiricus* and *Elymus dahuricus* had a higher abundance of *Bacillus* (19 and 11% respectively), and the *Elymus dahuricus* was also enriched with *Sphingomonas* (25%).

Differences in fungal communities across forages

Fungal communities of each forage seed also had a different proportion of shared fungal taxa; however, there was a greater significant difference in their relative abundance than was seen in bacteria. Fungal communities were dominated by different taxa at the class and order levels. The main fungal community OTUs were identified as *Ascomycota* (23~96%, per sample), *Basidiomycota* (2~11%) and other unclassified taxa (1~70%). *Ascomycota* was dominant in most of the forage seeds, especially in *Elymus sibiricus* (96%) and *Elymus dahuricus* (94%), while unclassified fungi were only abundant in

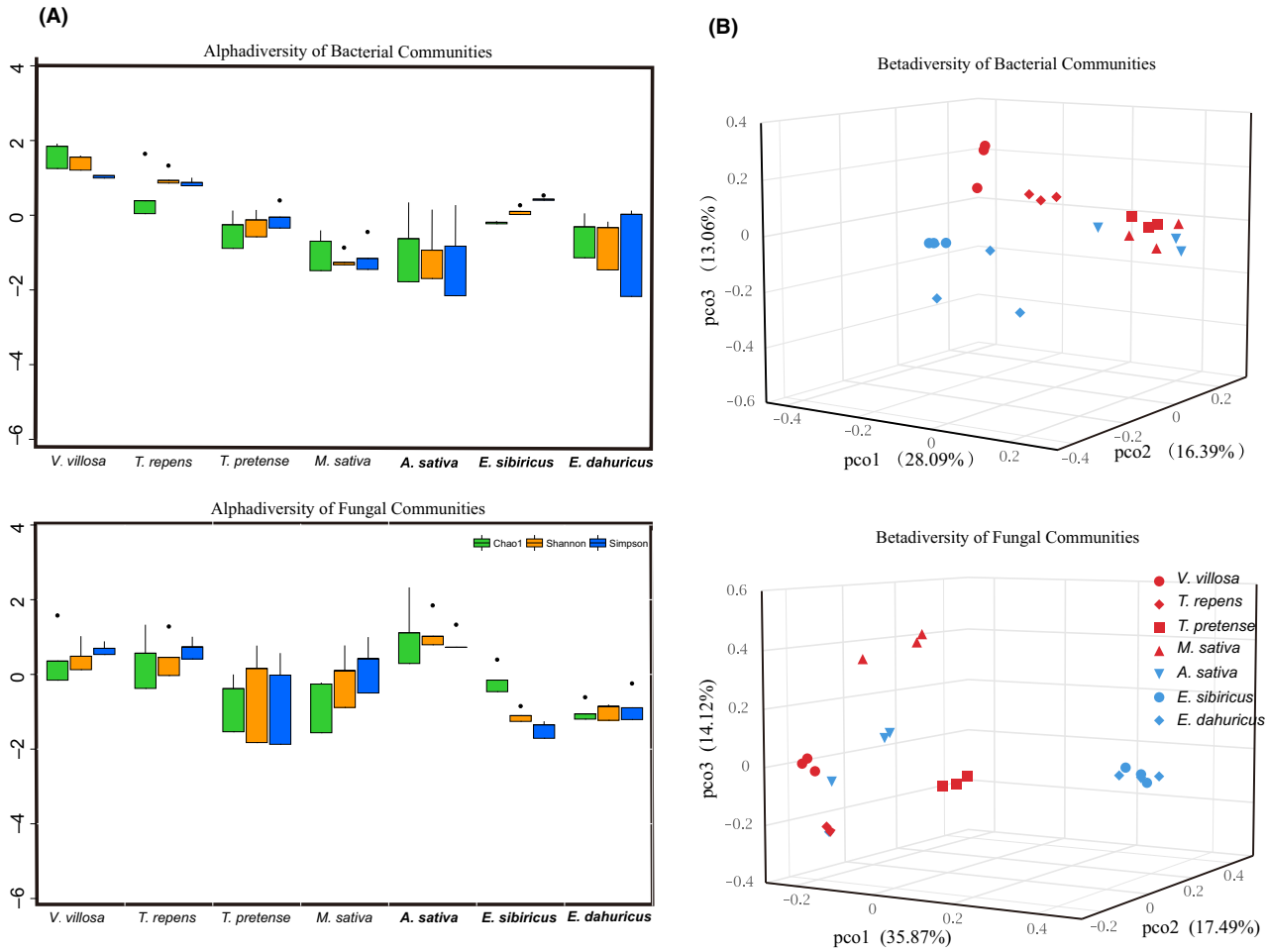

Fig. 2. The Alpha (A) and beta (B) diversity of the bacterial and fungal communities of each seed. Chao1 (green), Simpson's (orange) and Shannon indices (blue) are presented as indicated in (A). *Leguminous* (red) and *Gramineous* (blue) forages are presented as indicated in (B) The four legumes (*Vicia villosa*, *Trifolium repens*, *Trifolium pretense* and *Medicago sativa*) and three gramineous forage species (*Avena sativa*, *Elymus sibiricus* and *Elymus dahuricus*) were abbreviated in the figure. The beta diversity was based on the Bray–Curtis distance of OTUs.

Leguminous seeds, in particular *Trifolium pretense* (70%) and *Medicago sativa* (49%). At the class level, *Ascomycota* was dominated by *Leotiomycetes* (3~74%), *Dothideomycetes* (9~50%) and *Saccharomycetes* (5~33%). At the order level, *Helotiales* (2~73%) from *Leotiomycetes* was the most abundant, followed by *Saccharomycetales* (5~33%) from *Saccharomycetes*, *Pleosporales* (3~43%) and *Capnodiales* (2~23%) from *Dothideomycetes* (Table 1).

Of the fungal OTUs, 23, 45 and 68% were identified at the genus, family and order levels respectively (Fig. S2B), and a circos plot was made using the order-level information (Fig. 4). In *Vicia villosa*, *Candida* was the most abundant *genera* (33%) seeds. *Trifolium repens* also contained *Candida* (14%) and a large proportion of *Helotiales* (41%, *Sclerotiniaceae* dominated). *Trifolium pratense* had a large amount of unidentified fungi (70%). *Medicago sativa* was dominated by *Capnodiales* (23%), of which the *Mycosphaerellaceae* was the most abundant. *Avena sativa* was enriched with both *Candida* (17%) and *Alternaria* (18%). *Elymus sibiricus* and *Elymus dahuricus* were also enriched with *Helotiales* (69 and 59% respectively, *Helotiaceae* dominated). The unidentified OTUs in each species were dominated by different OTU, for example, OTU_2 in *Vicia villosa* (29%), OTU_9 in *Trifolium repens* (16%), OTU_5 in *Trifolium pratense* (55%) and OTU_4 in *Medicago sativa* (38%).

Bacterial community functions predicted by Tax4Fun

To assess how variation in bacterial communities may influence functional diversity in the seeds, Tax4Fun was used to predict the functional properties of the taxa units detected by 16S rRNA gene analysis. The bacterial

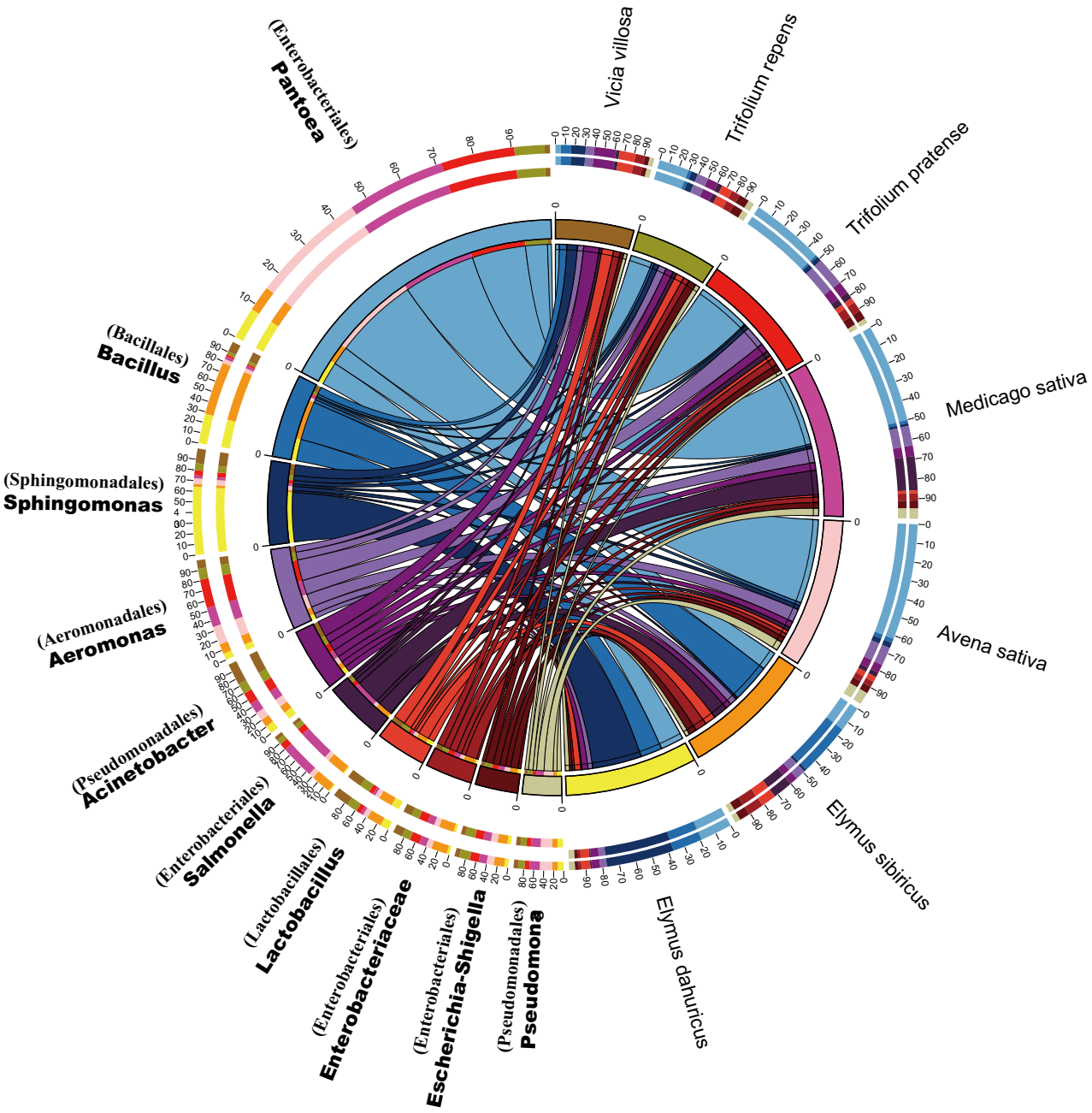

Fig. 3. The relative proportion of abundant bacterial taxa in each forage seed. The left side of the figure shows the ten most abundant genera in bacterial communities with the order name in brackets, and the right sides shows the different seed samples. The inner ring colours represent different taxa or seed samples, and the corresponding outer ring shows their proportion.

communities of *Trifolium repens* and *Trifolium pretense* were predicted to have similar function, as were the *Medicago sativa* and *Avena sativa*. Energy and amino acid metabolism functions were found to be significantly higher in the *Vicia villosa* community compared to other seeds, and nucleotide metabolism functions were significantly more abundant in *Trifolium repens* and *Trifolium pretense* communities (Fig. 5). With the exception of ABC transporters related functions, *Elymus* (*Elymus sibiricus* and *Elymus dahuricus*) communities were predicted to have significantly lower functions relating to many metabolic pathways compared to other seeds (Table S1). We also compared the abundance of six KEGG Ortholog (KO) profiles related to plant growth-promoting (PGP) and spore formation traits. The predicted abundances of enzyme-encoding genes involved in

Table 1. The relative abundance of dominant bacterial and fungal taxa (From phylum to order level).

Taxonomic assignment	*Vicia villosa*	*Trifolium repens*	*Trifolium pratense*	*Medicago sativa*	*Avena sativa*	*Elymus sibiricus*	*Elymus dahuricus*
Bacteria							
Proteobacteria [a]	**58%**	**60%**	**77%**	**90%**	**86%**	**54%**	**71%**
GammaProteobacteria[b]	34%	43%	**63%**	**80%**	**74%**	45%	28%
Enterobacteriales[c]	13%	25%	**40%**	**59%**	**52%**	32%	16%
Pseudomonadales[c]	10%	7%	7%	8%	8%	5%	5%
Aeromonadales[c]	3%	4%	**11%**	7%	7%	4%	2%
AlphaProteobacteria[b]	**19%**	7%	3%	3%	5%	2%	**31%**
Sphingomonadales[c]	7%	3%	2%	1%	3%	1%	**25%**
BetaProteobacteria[b]	3%	9%	7%	6%	6%	6%	10%
Burkholderiales[c]	2%	4%	3%	2%	3%	3%	8%
Firmcutes [a]	**25%**	16%	10%	5%	10%	**41%**	**24%**
Bacilli[b]	**19%**	11%	7%	5%	8%	**38%**	**21%**
Bacillales[c]	9%	3%	2%	3%	4%	**25%**	**15%**
Lactobacillales[c]	**10%**	8%	5%	2%	4%	12%	7%
Fungi							
Unidentified Fungi [a]	**39%**	**23%**	**70%**	**49%**	10%	2%	1%
Ascomycota [a]	**56%**	**71%**	**23%**	**46%**	**78%**	**96%**	**94%**
Leotiomycetes[b]	8%	**42%**	8%	3%	7%	**74%**	**63%**
Helotiales[c]	7%	**41%**	7%	2%	6%	**73%**	**62%**
Dothideomycetes[b]	14%	12%	9%	**33%**	**50%**	12%	21%
Pleosporales[c]	10%	8%	3%	9%	**43%**	7%	3%
Capnodiales[c]	2%	3%	5%	**23%**	5%	4%	6%
Saccharomycetes[b]	**33%**	**16%**	5%	9%	**19%**	7%	9%
Saccharomycetales[c]	**33%**	**16%**	5%	9%	**19%**	7%	9%
Basidiomycota [a]	4%	5%	7%	5%	**11%**	2%	5%

a,b,c. The OTU designation was performed at phylum, class and order level respectively.
Values with a high percentage were highlighted in bold.

general PGP and spore formation traits were greater in *Elymus* seeds than the others (Table S2).

Significant cooccurrence and coexclusion relationships among the bacterial and fungal OTUs

To understand how microbial species interact with each other, a microbial interaction network for the abundant bacterial OTUs (Abundance over 0.2%) in all samples was generated, and only 75 of 80 OTUs showed significant interactions (Pearson correlation coefficient > 0.5; $P < 0.05$) (Fig. 6). Taxa involved in these interactions were, in order of relative abundance, Proteobacteria (66.7%) > Firmicutes (24%) > *Bacteroides* (4.0%) > *Actinobacteria* (2.7%). Among the positive interactions observed, a strong cooccurrence relationship was shown with some members of *Sphingomonas*1, 2, 3, *Ralstonia*, *Burkholderia*, *Hyphomicrobiaceae* and *Methylobacterium*. We also found that some of the highly abundant OTUs were more likely to exhibit cooccurrence with members in same phylum and coexclusion with members of different phyla (Fig. 6). In *Proteobacteria*, the OTU *Pantoea*1 was positively correlated with five OTUs in same phylum (*Pantoea* 2, 3, *Aeromonas*1, *Haemophilus*1) and negatively correlated with members of different phyla, including *Firmicutes* (*Lactobacillus*1, 2, *Enterococcus, Rummeliibacillus and Lysinibacillus*) and *Actinobacteria* (*Rothia*). The presence of *Aeromonas*1 correlated positively with microbes of the same phylum and negatively with members of other phyla. *Bacillus*1 belonging to *Firmicutes* was positively correlated with other *Firmicutes* (including seven OTUs).

In fungal OTUs, 39 out of 42 exhibited significant interactions (Fig. S3), dominated by *Ascomycota* (66.7%), *Basidiomycota* (20.5%) and unidentified fungi (12.8%). Most of the significant interactions were positive (72/74), and the highly abundant taxa were not only cooccurrence with members of the same phylum, but also with members of other phyla.

Isolated Pantotea *and* Bacillus *differentially promote effects on plants*

From the high-throughput 16S rRNA gene data, *Pantoea* and *Bacillus* were the two most abundant taxa detected in the analysed bacterial communities. *Pantoea* were highly abundant in all the other seeds except for *Vicia villosa*, and *Bacillus* were only significantly enriched in *Elymus sibiricus* and *Elymus dahuricus*. Reportedly, most of *Pantoea* and *Bacillus* strains were beneficial to plant growth (plant growth-promoting bacteria, PGPB). We then tested whether the strains on the seeds could be isolated and whether they have beneficial effects on plant germination and growth. First, we undertook efforts to culture *Pantoea* and *Bacillus* from the seven seeds. After incubating the forage seeds in

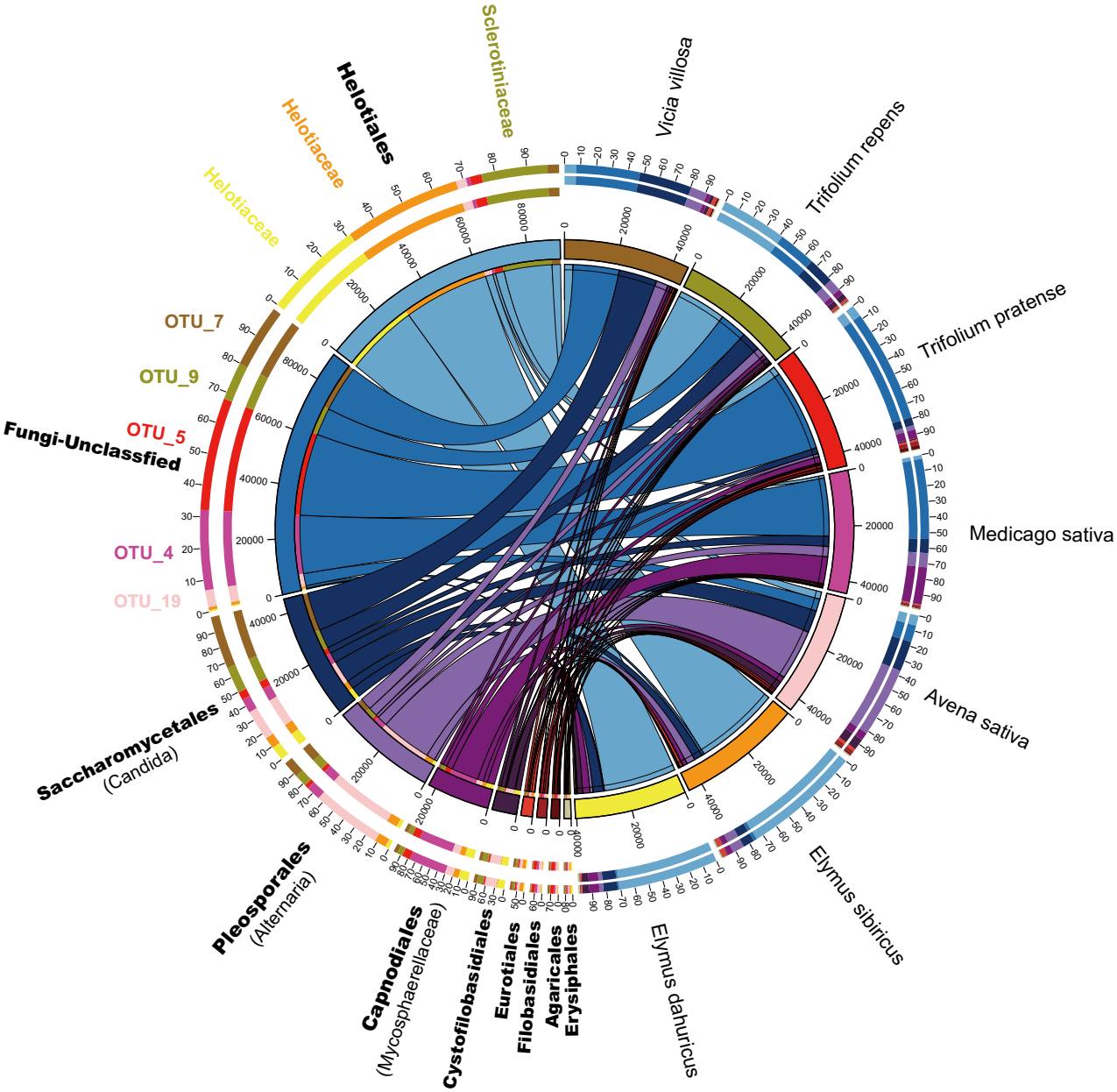

Fig. 4. The relative proportion of abundant fungal taxa in each forage seed. The left side of the figure shows ten most abundant orders in fungal communities, with the genus name in brackets, and the right shows the seed samples. The different colours of the inner ring represent different taxa or seed samples, and the corresponding outer ring shows their proportion. When the order present in some samples is dominated by different taxa, the dominant OTUs or family name are marked by the samples' corresponding colour.

LB solid medium, separate colony morphologies of ten different bacterial strains were observed, including five *Bacillus*, three *Pantoea*, one *Staphylococcus* and one *Salmonella*. Based on the BLAST results of 16S rRNA gene sequences and the forage species they were isolated from, the ten strains were named as *Bacillus subtilis* Es-1 ('Es' means isolated from seeds of *Elymus sibiricus*), *Bacillus licheniformis* Ed-2, *Bacillus atrophaeus* Ed-3, *Bacillus safensis* Es-4, *Bacillus cereus* As-5, *Staphylococcus pasteuri* Tp-1, *Salmonella.sp* Ms-1, *Pantoea ananatis* Es-1, *Pantoea agglomerans* Tr-2 and *Pantoea agglomerans* Ed-3 respectively. Most of these strains have a positive effect on *Medicago sativa* germination under salt stress, of which *Bacillus subtilis* Es-1 and *Pantoea agglomerans* Ed-3 performed best (Fig. S4). These two strains were then chosen to test whether their positive effects could be affected by the seed-resident microbiome.

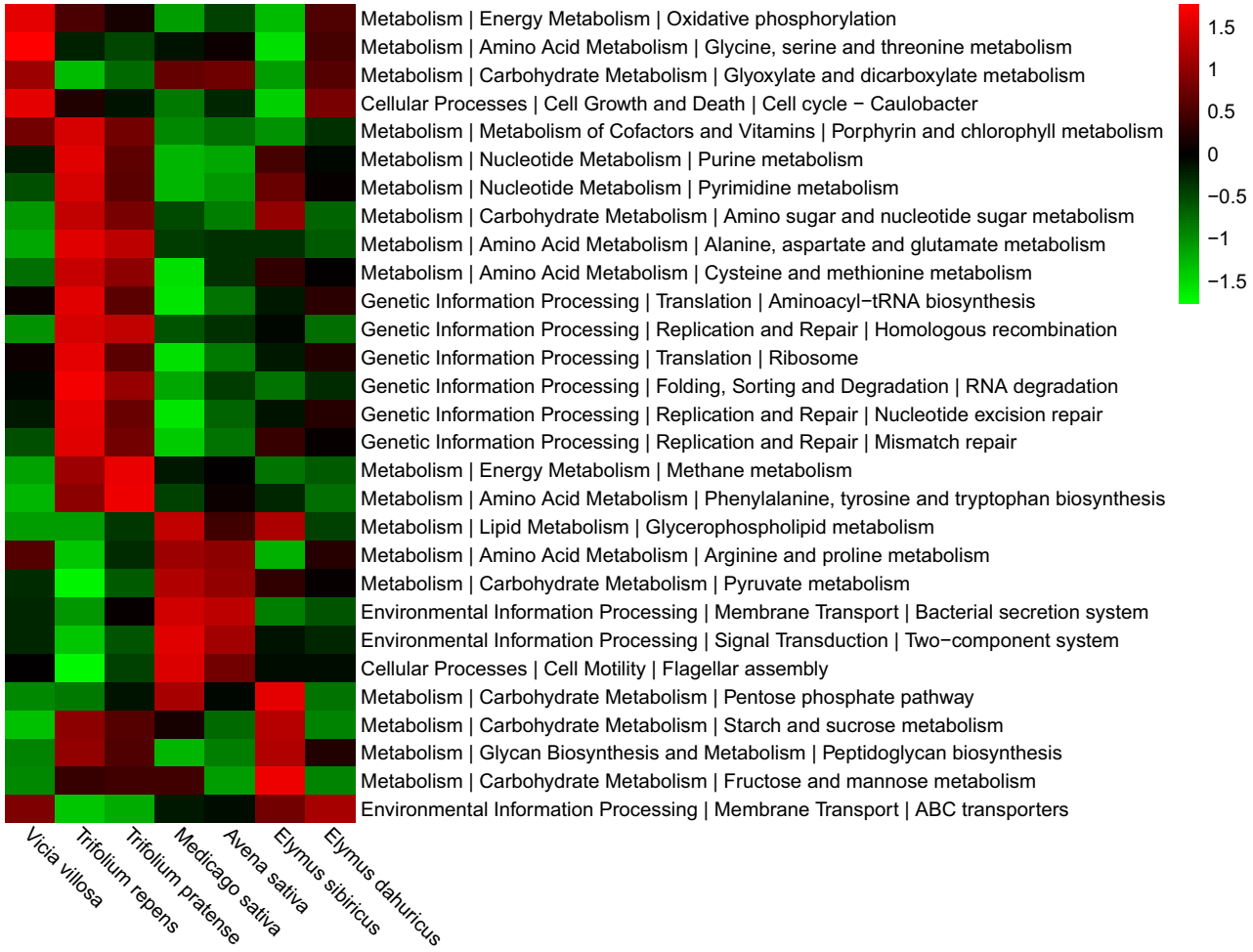

Fig. 5. Relative proportions of predicted function of 16S rRNA genes at KEGG level 3. All of the functions of genes with an abundance > 1%. The colour indicates the Z score of relative predicted abundance, from high (red) to low (Green).

Two gramineous (*Elymus sibiricus* and *Elymus dahuricus*) and two leguminous forages (*Vicia villosa* and *Medicago sativa*) were selected as the test plants according to the different abundance of *Pantoea* or *Bacillus*. The surface-sterilized seeds were soaked in sterile water (Control) or sterile water containing 10^7 bacteria mL^{-1} for 4 h at 28 °C, separately. We saw that the two bacterial strains differently influenced seed germination and seedlings' growth of the four forages (Fig. 7A–D). External incubation of *Bacillus subtilis* Es-1 significantly increased the germination rate ($P < 0.001$) and promoted the seedling growth of *Vicia villosa* ($P < 0.01$). The germination and biomass of *Medicago sativa* were also slightly promoted by *B. subtilis*. However, we observed that *B. subtilis* Es-1 had a significant inhibitory effect on the germination and seedling growth of *Elymus sibiricus* and *Elymus dahuricus*, with inhibition more pronounced in *Elymus sibiricus*. The other strain *Pantoea agglomerans* Ed-3 also promoted germination ($P < 0.05$) and seedling growth ($P < 0.01$) of *Vicia villosa*, but the effect was not as significant as that seen with *B. subtilis Es-1*. While it has a negative effect on the germination and growth of *Medicago sativa*, *Elymus sibiricus* and *Elymus dahuricus*, it is especially pronounced in *Elymus sibiricus* ($P < 0.001$)(Fig. 7E and F).

Discussion

Although these forage seeds were endophyte rich and had different microbiome structures, an extensive overlap exists across the four leguminous and three gramineous forages. At the phylum level, *Proteobacteria* and *Ascomycota* were the dominant two phyla in all forage seeds. It has been reported that many taxa in the two phyla like *Pantoea* and *Candida* are conserved in many plant seeds (Links *et al*., 2014; Barret *et al*., 2015). We only detected higher abundance of *Firmicutes* in forage species with higher stress resilience, like *Vicia villosa*

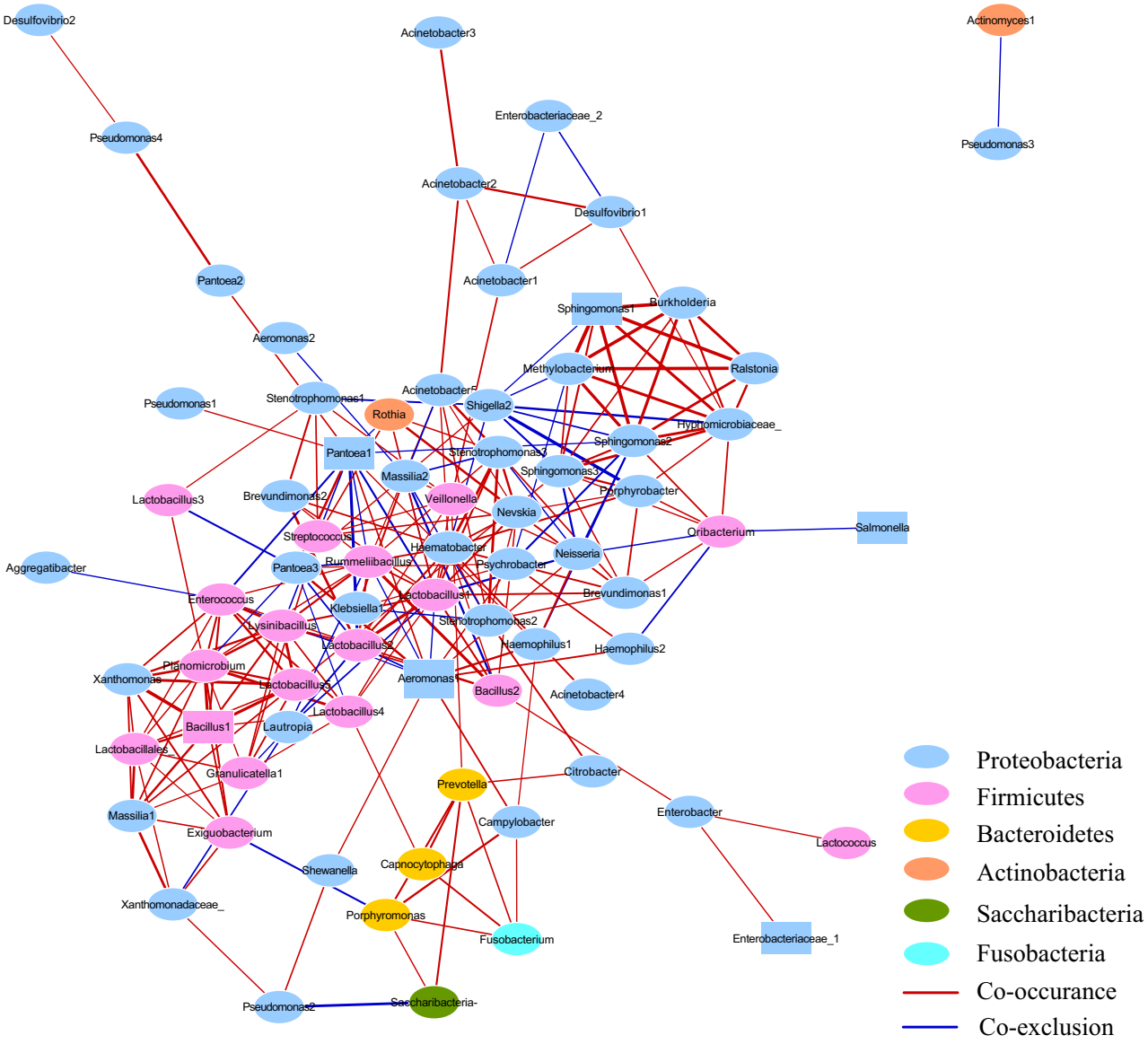

Fig. 6. Significant cooccurrence and coexclusion relationships among the bacterial microbiome of the forage seeds. Each node represents a bacterial OTU (abundance > 0.2%), described at the genus-level. In cases where the OTU was not assigned on a genus level, higher-level taxonomic groups have been shown and labelled as '_' after the name. When several OTUs are assigned to the same taxa, the numbers (1~5) are added to the name to differentiate between the nodes. Nodes colour corresponds to phylum, and rectangular nodes represent abundant OTUs (Abundance > 4%). Only significant interactions are shown (Pearson correlation coefficient > 0.5; $P < 0.05$). Edge width is proportional to the absolute value of the Pearson correlation coefficient, and colour indicates the sign of the association (red positive, blue negative).

(25%), *Elymus sibiricus* (41%) and *Elymus dahuricus* (24%). This agrees with other studies which have shown higher levels of *Firmicutes* in other stress-tolerant plants such as *Agave* and *Quinoa* (Coleman-Derr *et al*., 2016; Pitzschke, 2016). All of which suggests that *Firmicutes* may have an important role in plant stress resistance.

The beta diversity data revealed that the seven seeds' fungal communities were clustered more distinctively by host genotypes than bacteria (Fig. 2B; Fig. S1). This was consistent with microbiome study in *Brassicaceae* (Barret *et al*., 2015) and sunflower (Leff *et al*., 2017), where host genotypes were shown to have a stronger effect on fungal microbiota than bacteria. Genetic background aside, the seven forage seeds in our study also had different seed weight, morphological structure and geographical location. All factors have been reported to influence seed microbiome structure (Khalaf and Raizada, 2016). *Avena sativa* seed was significantly different from *Elymus sibiricus* and *Elymus dahuricus* in seed weight and morphological structure (Table 2; Fig. S5), which may partly explain the differences between their microbial community structures.

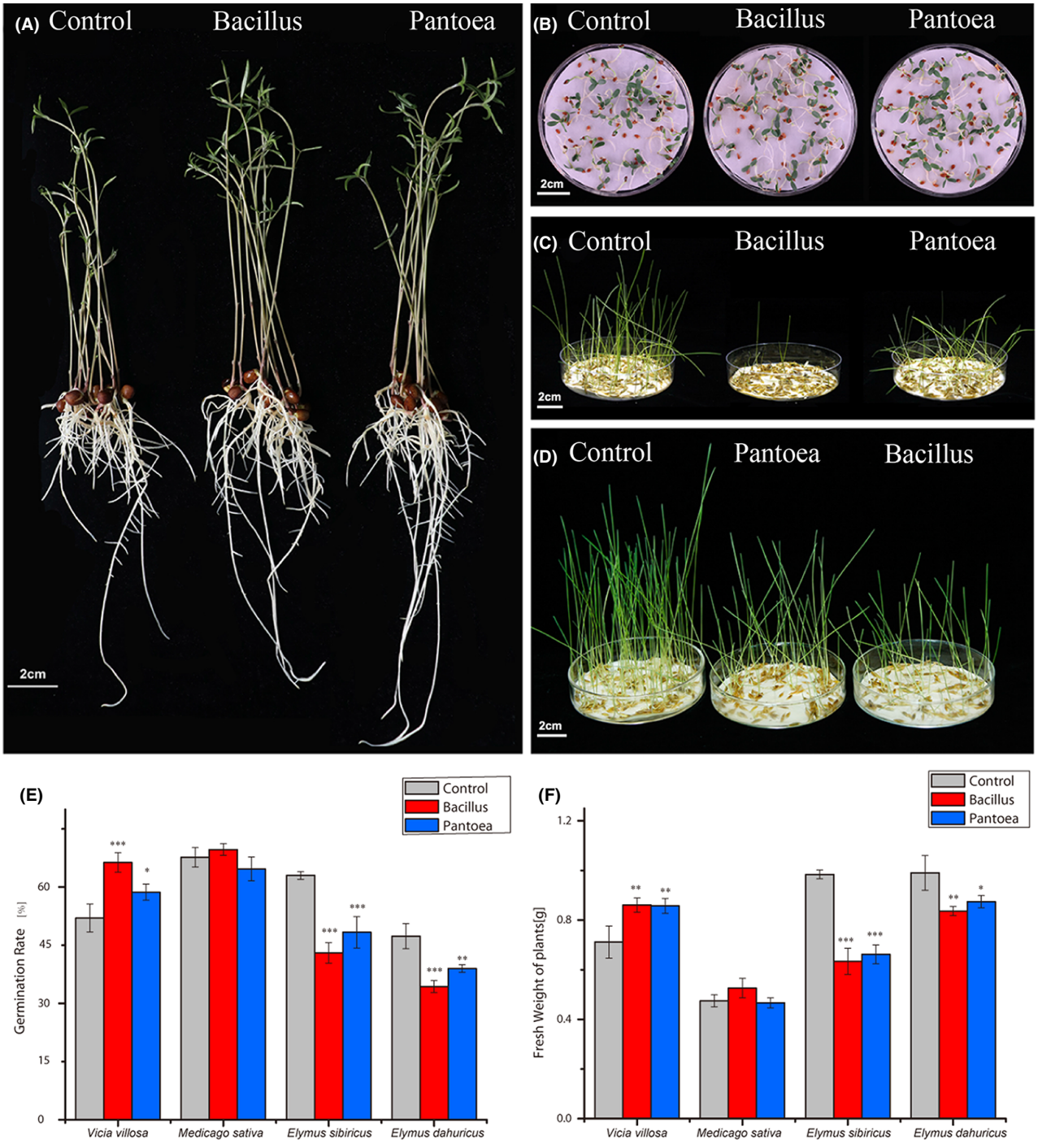

Fig. 7. Effect of the two isolated bacterial strains on the germination rate and seedling fresh weight of forages. *Vicia villosa* (A) and *Medicago sativa* (B) seedlings at one week old, and *Elymus sibiricus* (C) and *Elymus dahuricus* (D) seedlings at two weeks old. The statistical results of germination rate (E) and seedling fresh weights (F) are shown. '*Bacillus*' means treatment with *Bacillus subtilis* Es-1 and '*Pantoea*' means treatment with *Pantoea agglomerans* Ed-3. The fresh weight of *Vicia villosa* was the average weight of three plants in one replicate group, and the fresh weight of *Medicago sativa*, *Elymus sibiricus* and *Elymus dahuricus* was the average weight 30 plants in one replicate group. Data expressed as the geometric mean ± standard deviation. Tukey HSD *t* test was used to determine significance (* significant at $P < 0.05$, ** significant at $P < 0.01$, *** significant at $P < 0.001$).

These ecologically and geographically diverse seeds have their own unique microbiota; however, the abundance of OTUs specific to each seed was low. Therefore, the differences in the bacterial or fungal communities come mainly from the relative abundances of the shared OTUs, rather than the presence or absence

Table 2. Seed samples analysed in this study.

Samples	Family	Geographical origin	Thousand Kernel Weight g^{-1}
Vicia villosa	*Leguminosae*	Yunnan, China	62.49
Trifolium repens	*Leguminosae*	Unknown, Australia	0.67
Trifolium pratense	*Leguminosae*	Oregon, USA	1.89
Medicago sativa	*Leguminosae*	Oregon, USA	1.98
Avena sativa	*Poaceae*	Sichuan, China	32.1
Elymus sibiricus	*Poaceae*	Sichuan, China	4.22
Elymus dahuricus	*Poaceae*	Sichuan, China	4.18

of specific OTUs. Many shared OTUs enriched in these forage seeds were reported to promote plant growth and improve resilience to biotic and abiotic stresses. *Pantoea* are IAA (indole-3-acetic acid) producing, fungal antagonistic and osmotic tolerant endophytes (Links *et al*., 2014; Walitang *et al*., 2017). We found that *Pantoea* were dominant in *Avena sativa* (42%), *Medicago sativa* (36%), *Trifolium pratense* (27%), *Elymus dahuricus* (12%), *Trifolium repens* (11%) and *Elymus sibiricus* (9%). Although the *Vicia villosa* seeds had lower *Pantoea* (2%) abundance, it contained a wealth of other beneficial taxa including *Acinetobacter* (8%) (Martinez-Rodriguez *et al*., 2019) and *Sphingomonas* (5%). *Candida* was found in high abundance in *Vicia villosa* (31%), *Avena sativa* (17%) and *Trifolium repens* (14%) and was reported to be a saprotroph and unlikely to be a pathogen (Nguyen *et al*., 2016). As with native forages, the candidate legume forage seeds also contained several potentially beneficial taxa, which suggest that they may have the potential to adapt to the harsh environment of the Qinghai–Tibet Plateau.

High amounts of *Bacillus* and *Helotiaceae* were only found in *Elymus sibiricus* and *Elymus dahuricus*, and *Elymus dahuricus* was also shown to have high levels of *Sphingomonas* (25%). It has been reported that many *Bacillus* and *Sphingomonas* species are considered beneficial taxa (Yang *et al*., 2014; Martinez-Rodriguez *et al*., 2019). Tax4fun prediction also indicated a lower rate of activity in many metabolic pathways and a higher spore-forming ability within the bacterial cells of *Elymus sibiricus* and *Elymus dahuricus*. Bacteria may be able to buffer impacts from changing environment by reducing their metabolic costs and forming spores (Jones and Lennon, 2010; Lennon and Jones, 2011). Most *Helotiales* members are presumed to be non-pathogenic (probable saprotroph–symbiotroph) (Nguyen *et al*., 2016). The excellent stress resistance characteristics of Quinoa were partly conferred by the endophytes transmitted from seeds, especially *Bacillus.sp* (Pitzschke, 2016). Therefore, the high-stress tolerance of *Elymus sibiricus* and *Elymus dahuricus* in Qinghai–Tibet Plateau may be partly due to the high abundance of beneficial taxa like *Bacillus*, *Pantoea* and *Helotiaceae*.

It has been previously reported that a high-abundance bacteria and fungi does not necessarily promote plant growth directly, but is more likely to participate in organizing the assembly of plant-associated microbiomes to improve host growth (Toju *et al*., 2018). According to the microbial interaction networks, some bacterial taxa were more likely to cooccur with members of the same phylum, but coexclude with members of different phyla (Fig. 6). A pattern was more pronounced among high-abundance OTUs. One OTU belonging to the putative plant pathogen *Ralstonia* was reported to positively correlate with the beneficial *Burkholderiaceae* in the *Brassica napus* seed microbiome (Rybakova *et al*., 2017). *Ralstonia* was also positively correlated with beneficial taxa like *Sphingomonas*1/2/3 and *Burkholderia* in our results. Some bacterial taxa may be involved in the recruitment of beneficial genera and are more likely to recruit bacteria from the same phylum.

In our study, three *Pantoea.sp* and five *Bacillus.sp* were isolated from seeds, and all showed positive effects on *Medicago sativa* germination rate under salt stress. Many *Bacillus* and *Pantoea* species were reported to have beneficial effects on plants, including IAA production, plant growth promotion, antifungal and osmotic stress tolerance (Malfanova *et al*., 2011; Martinez-Rodriguez *et al*., 2019). These strains may have more biotechnological potential in sustainable agriculture. Our study preliminary tested the salt-tolerant and growth-promoting functions of the isolated strains, and the other functions need to be further studied. However, *Bacillus subtilis* Es-1 showed negative effects when the host microbiomes were rich in *Bacillus*, as was the case with *Pantoea agglomerans* Ed-3 strains. The effect of the bacterial inoculants on the host may largely depend on the interactions within and between indigenous microbiomes (Castro-Sowinski *et al*., 2007). It was also reported in *Brassica napus* that community diversity and the relative abundance of specific taxa can influence the effects of symbionts and pathogens (Rybakova *et al*., 2017). Thus, the isolated *Pantoea* and *Bacillus* can promoted the seed germination under salt stress, while the beneficial effects may be counteracted by a high abundance of specific taxa within the seed-resident microbiome.

In conclusion, the four cold-tolerance legumes and three Qinghai–Tibet Plateau gramineous grasses shared a high number of taxa, including *Bacillus*, *Pantoea*, *Candida* and *Helotiales*, but the relative proportion of these taxa was different in each seed. The fungal communities clustered more distinctively according to host genotypes than bacterial. As with native forages, the four legume forage seeds also contained several potentially beneficial

taxa and may have the potential to adapt to the harsh environment of the Qinghai–Tibet Plateau. The high-stress tolerance of *Elymus sibiricus* and *Elymus dahuricus* in Qinghai–Tibet Plateau may be partly due to the high abundance of taxa like *Bacillus*, *Pantoea* and *Helotiaceae*. Some bacterial taxa may be involved in the recruitment of beneficial genera and are more likely to recruit bacteria from the same phylum. The potentially beneficial strains *Pantoea* and *Bacillus* could be isolated from the seeds and can promote the seed germination under salt stress. However, the beneficial effects may be counteracted by a high abundance of specific taxa within the seed-resident microbiome; thus, it is better to use probiotics that seeds naturally lack, rather than those they already have. The structure of the seed microbiome could be an interesting biomarker for breeding strategies. Nevertheless, managing species-rich communities of plant-associated microbiomes remains a major challenge and our research provides the biotechnological potential of high-throughput sequencing for screening beneficial strains, analysing the interaction between microorganisms and plants, and provides further theoretical guidance to make full use of microbial resources to improve the sustainability of global agriculture.

Experimental procedures

Seed sources

Seven different forages were used in this study (Table 2). The gramineous seeds including *Avena sativa*, *Elymus sibiricus* and *Elymus dahuricus* were collected from Hongyuan County, Sichuan Province, China (elevation 3500~3600 m, 31°50'~33°22'°N, 101°51'~103°23'°W), located in the eastern Qinghai–Tibet Plateau. The leguminous seeds including *Vicia villosa*, *Trifolium repens*, *Trifolium pretense* and *Medicago sativa* were cultivated in different countries (China, Australia and USA) and then purchased from Beijing Baxter Grass Company (Table 2). All of the seeds were collected in the summer of 2017 and stored separately in plastic bags at −20 °C until analysis. Host phylogenetic analysis was determined through the analysis of sequence of genes rbcL (Rubisco large subunit) and matK (tRNA-Lys) intron that were downloaded from NCBI (Naylor *et al*., 2017).

Sample preparation

Ten grams of each seed were surface-sterilized with 4% sodium percarbonate ($Na_2CO_3{\cdot}1.5H_2O_2$, aladdin^R) with shaking in an orbital shaker at 180 rpm for 15 to 30 min (time determined based on differences in the texture of the seed coat). The bleach was then aspirated, and seeds were rinsed three times with sterile water. To check surface sterility, 30 seeds were placed on top of an LB plate. On the other hand, after water removal, seeds were physically ground into powder in a 75% ethanol-sterilized grinding machine (Royalstar GR150A) for 1–3 min. Seed powder was packed into a 2 ml centrifuge tube and stored at −20 °C for later use.

DNA extraction, PCR and sequencing

Total genomic DNA from 150 mg seed samples (prepared as described above) was extracted using a Quick-DNA Plant/Seed Miniprep Kit (Zymo^R, Catalog No. D6020) according to manufacturer's instructions. DNA concentration and quality were checked using a NanoDrop Spectrophotometer. Each sequencing run was performed with three biological replicates and three technical replicates. The V4 region of 16S rRNA gene and the ribosomal internal transcribed spacer 2 (ITS2) were PCR-amplified to assess bacterial and fungal diversity respectively (Caporaso and Gordon, 2011; Tedersoo *et al*., 2015). For 16S rRNA gene amplification, the phosphorylated locked nucleic acid primer pairs PLNAM (GTCGAACGTTGT TTTCGGp/CTTCACCCCAGTCGAAGAp) and PLNAP (GTCGAACGGGAAGTGGTp/CTTCACTCCAGTCGCAA GCp) were used to block amplification of the host plant plastid and mitochondrial sequences, which increased the proportion of prokaryotic reads of the seed endosphere. Primer: 515F (GTGYCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) were used to amplify the V4 region of 16S rRNA gene and Primer: ITS3_KYO2 (GATGAAGAACGYAGYRAA) and ITS4 (TCCTCCGCTT ATTGATATGC) were used to amplify the ITS2 region. Each seed sample was amplified using the specific primer with 12 nt unique barcode.

The PCR mixture (50 μL) contained 2x PCR buffer, 1.5 mM $MgCl_2$, each deoxynucleoside triphosphate (dNTP) at 0.4 μM, each primer at 1.0 μM, 0.5 U of KOD-Plus-Neo (TOYOBO) and 10 ng template DNA. The PCR amplification programme consisted of an initial denaturation step of 94 °C for 1 min, followed by 30 cycles of denaturation at 94 °C for 20 s, annealing at 55 °C (16S rRNA gene) or 48 °C (ITS) for 30 s, elongation at 72 °C for 30 s and a final extension at 72 °C for 5 min. PCR products from different samples were pooled with equimolar amounts, and the library was subjected to paired-end sequencing (2 × 250 bp) using the Illumina Hiseq apparatus at Rhonin Biosciences Co., Ltd. All quality sequence files supporting the findings of this article are available in the NCBI Sequence Read Archive (SRA) under the BioProject IDs PRJNA578890 and PRJNA578958.

Data processing and statistical analyses

The sequences were analysed according to Usearch (http://drive5.com/uparse/) and QIIME (Caporaso *et al*.,

2010) pipeline. Paired-end reads from the original DNA fragments were merged using FLASH (Magoč and Salzberg, 2011). Then, sequences were assigned to each sample according to the unique barcode. Sequences were clustered into operational taxonomic units (OTUs) at 97% identity threshold using UPARSE algorithms (Edgar, 2013). To obtain the taxonomic information of the OTUs, representative sequences of each OTU were generated and aligned against the SILVA and UNITE databases using the uclust classifier in QIIME for 16S rRNA gene and ITS2 respectively. We picked representative sequences and removed potential chimeras using Uchime algorithm. In case of the influences of sequencing depth on community diversity, the OTUs table was subsampled to the lowest number of read counts for further analysis. All data analyses were performed using R7 or Python (https://www.python.org/). Weighted and unweighted Unifrac distances were calculated in GUniFrac. Calculation of community parameters including Chao1 richness, Simpson's index D and the Shannon–Weiner index H' was performed using mothur. Principal coordinate analysis (PCoA) was performed to assess the beta diversity based on Bray–Curtis distance. Possible bacterial community functions were predicted with Tax4Fun (Asshauer *et al*., 2015), and genes related to plant growth-promoting (PGP) and sporulation were selected based on study of grassland soils and Grapevine rootstocks (Griffith *et al*., 2017; Marasco *et al*., 2018). A Kruskal–Wallis rank sum test was performed to determine the significance of difference in alpha diversity and taxa among different groups. To determine whether there was significant distinct among different groups, permutational multivariate analysis of variance (PERMANOVA) was performed based on the dissimilarity matrix. Differences in the relative abundances of genera between samples were determined using a Student two-sample t tests.

Isolation and identification of bacteria from forage seeds

We examined whether the strains in the seeds could be isolated and whether they affected plant germination and growth. About 0.2 g of the seed powder described above was added to a triangular flask with 20 ml of sterile water and 5 g of sterile glass beads (3.5 mm diameter). The seed microbiota was incubated at 28 °C in an orbital shaker (180 rpm) for 4 h. Ten-fold serial dilutions of powdered seed suspensions (10× and 100×) were streaked on LB solid plates (10 g l^{-1} tryptone, 5 g l^{-1}, yeast extract and 10 g l^{-1} NaCl, pH 7.2, 15 g l^{-1} agar) and incubated for 3 days at 28 °C. Morphologically unique bacterial colonies from each plate were selected and streaked onto fresh plates to purify them and cultured in LB broth for glycerol stocks and DNA extraction. Genomic DNA of each isolate was extracted, and PCR was performed using the 16s rRNA primers 27F/1492R (Delong, 1992). Sanger sequencing of the PCR product was carried out in sequencer ABI 3730XL. The obtained 16S rRNA gene sequences were aligned using both BLAST analysis in the EzTaxon server (https://www.ezbioc loud.net/) (Ok-Sun *et al*., 2012) and NCBI BLAST Resources (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The 16S rRNA genes nucleotide sequences of the two isolates used in our study were deposited to the GenBank® database under accession numbers MN595066 and MN595067.

Plant growth promotion assay

The influence of the microbiome on the growth-promoting effects of selected bacterial strains was assayed by seed inoculation experiments (Sheibani-Tezerji *et al*., 2015). The germination rate and the seedling fresh weights were calculated for assessing the effect of growth promotion. The seeds of *Vicia villosa*, *Medicago sativa*, *Elymus sibiricus* and *Elymus dahuricus* were surface-sterilized as above. Bacterial strains were grown in LB medium for 24 h at 28 °C and cell density determined by colony forming unit (CFU) analysis. Bacterial inoculation was performed by soaking seeds in water containing 10^7 bacteria ml^{-1} for 4 h at 28 °C with control seeds soaked in sterile water. Seeds were then transferred to a petri dish with two layers of filter paper and 5 ml of sterile water, and the seed germination rate was calculated after 4 days, and *Medicago sativa*, *Elymus sibiricus* and *Elymus dahuricus* seedlings could be rooted in the filter paper and grow in the petri dish without cover, while the *Vicia villosa* seedlings were larger and could not be fixed on filter paper and were transferred into a seed germination pouch (PHYTOTC™, Size: 140 mm × 125 mm). Fresh seedlings weights were taken from 7-day-old *Vicia villosa* and *Medicago sativa* and 14-day-old *Elymus dahuricus* and *Elymus sibiricus*. The experiment was performed in triplicate. The significance of the differences in the germination rate and fresh weights between the control and treatment groups was calculated using a pairwise t test with independent samples in SPSS program version 20.0.

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Conflict of interest

None declared.

References

Andreozzi, A., Prieto, P., Mercado-Blanco, J., Monaco, S., Zampieri, E., Romano, S., *et al*. (2019) Efficient colonization of the endophytes Herbaspirillum huttiense RCA24 and Enterobacter cloacae RCA25 influences the physiological parameters of *Oryza sativa* L. cv. Baldo rice. *Environ Microbiol* **21:** 3489–3504.

Asshauer, K.P., Wemheuer, B., Daniel, R., and Meinicke, P. (2015) Tax4Fun: predicting functional profiles from metagenomic 16S rRNA data. *Bioinformatics* **31:** 2882–2884.

Barret, M., Briand, M., Bonneau, S., Préveaux, A., Valière, S., Bouchez, O., *et al*. (2015) Emergence shapes the structure of the seed-microbiota. *Appl Environ Microbiol* **81:** 1257.

Buchholz, F., Kostić, T., Sessitsch, A., and Mitter, B. (2018) The potential of plant microbiota in reducing postharvest food loss. *Microb Biotechnol* **11:** 971–975.

Caporaso, J.G., and Gordon, J.I. (2011) Global patterns of 16s rrna diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci USA* **108:** 4516–4522.

Castro-Sowinski, S., Herschkovitz, Y., Okon, Y., and Jurkevitch, E. (2007) Effects of inoculation with plant growth-promoting rhizobacteria on resident rhizosphere microorganisms. *FEMS Microbiol Lett* **276:** 1–11.

Cheesanford, J.C., Ii, M.M.W., Davis, A.S., and Sims, G.K. (2006) Do microorganisms influence seed-bank dynamics? *Weed Sci* **54:** 575–587.

Coleman-Derr, D., Desgarennes, D., Fonseca-Garcia, C., Gross, S., Clingenpeel, S., Woyke, T., *et al*. (2016) Plant compartment and biogeography affect microbiome composition in cultivated and native Agave species. *New Phytol* **209:** 798–811.

Compant, S., Clément, C., and Sessitsch, A. (2009) Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. *Soil Biol Biochem* **42:** 669–678.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D. & Costello, E.K., *et al*. (2010) QIIME allows analysis of high-throughput community sequencing data. *Nat Methods*, **7:** 335–336.

Delong, E.F. (1992) Archaea in coastal marine environments. *Proc Natl Acad Sci USA* **89:** 5685–5689.

Du, W., Hou, F., Tsunekawa, A., Kobayashi, N., Peng, F., and Ichinohe, T. (2019) Substitution of leguminous forage for oat hay improves nitrogen utilization efficiency of crossbred Simmental calves. *J Anim Physiol Anim Nutr* 1–12.

Edgar, R.C. (2013) UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods* **10:** 996.

Fukami, T. (2015) Historical contingency in community assembly: integrating niches, species pools, and priority effects. *Annu Rev Ecol Evol Syst* **46:** 1–23.

Goggin, D.E., Emery, R.J.N., Kurepin, L.V., and Powles, S.B. (2015) A potential role for endogenous microflora in dormancy release, cytokinin metabolism and the response to fluridone in Lolium rigidum seeds. *Ann Bot* **115:** 293.

Gond, S.K., Bergen, M.S., Torres, M.S., and White, J.F. Jr (2015) Endophytic *Bacillus* spp. produce antifungal lipopeptides and induce host defence gene expression in maize. *Microbiol Res* **172:** 79–87.

Griffith, J.C., Lee, W.G., Orlovich, D.A., and Summerfield, T.C. (2017) Contrasting bacterial communities in two indigenous Chionochloa (Poaceae) grassland soils in New Zealand. *PLoS One* **12:** e0179652.

Hallmann, J., Quadt-Hallmann, A., Mahaffee, W.F., and Kloepper, J.W. (1997) Bacterial endophytes in agricultural crops. *Can J Microbiol* **43:** 895–914.

Hardoim, P.R., van Overbeek, L.S., Berg, G., Pirttila, A.M., Compant, S., Campisano, A., *et al*. (2015) The Hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. *Microbiol Mol Biol Rev* **79:** 293–320.

Jones, S.E., and Lennon, J.T. (2010) Dormancy contributes to the maintenance of microbial diversity. *Proc Natl Acad Sci USA* **107:** 5881–5886.

Khalaf, E.M., and Raizada, M.N. (2016) Taxonomic and functional diversity of cultured seed associated microbes of the cucurbit family. *BMC Microbiol* **16:** 131.

Klaedtke, S., Jacques, M.A., Raggi, L., Préveaux, A., Bonneau, S., Negri, V., *et al*. (2016) Terroir is a key driver of seed-associated microbial assemblages. *Environ Microbiol* **18:** 1792–1804.

Lanza, M., Haro, R., Conchillo, L.B., and Benito, B. (2019) The endophyte Serendipita indica reduces the sodium content of Arabidopsis plants exposed to salt stress: fungal ENA ATPases are expressed and regulated at high pH and during plant co-cultivation in salinity. *Environ Microbiol* **21:** 3364–3378.

Leff, J.W., Lynch, R.C., Kane, N.C., and Fierer, N. (2017) Plant domestication and the assembly of bacterial and fungal communities associated with strains of the common sunflower, *Helianthus annuus*. *New Phytol* **214(1),** 412–423.

Lennon, J.T., and Jones, S.E. (2011) Microbial seed banks: the ecological and evolutionary implications of dormancy. *Nat Rev Microbiol* **9:** 119–130.

Links, M.G., Tigst, D., Tom, G.F., Hill, J.E., Hemmingsen, S.M., and Dumonceaux, T.J. (2014) Simultaneous profiling of seed-associated bacteria and fungi reveals antagonistic interactions between microorganisms within a shared epiphytic microbiome on Triticum and Brassica seeds. *New Phytol* **202:** 542–553.

Magoč, T. & Salzberg, S.L. (2011) FLASH: Fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* **27:** 2957–2963.

Malfanova, N., Kamilova, F., Validov, S., Shcherbakov, A., Chebotar, V., Tikhonovich, I., and Lugtenberg, B. (2011) Characterization of Bacillus subtilis HC8, a novel plant-beneficial endophytic strain from giant hogweed. *Microb Biotechnol* **4:** 523–532.

Marasco, R., Rolli, E., Ettoumi, B., Vigani, G., Mapelli, F., Borin, S., *et al*. (2012) A drought resistance-promoting

microbiome is selected by root system under desert farming. *PLoS One* **7:** e48479.
Marasco, R., Rolli, E., Fusi, M., Michoud, G., and Daffonchio, D. (2018) Grapevine rootstocks shape underground bacterial microbiome and networking but not potential functionality. *Microbiome* **6:** 3.
Martinez-Rodriguez, A., Macedo-Raygoza, G., Huerta-Robles, A.X., Reyes-Sepulveda, I., Lozano-Lopez, J., García-Ochoa, E.Y., *et al*. (2019) Agave seed endophytes: ecology and impacts on root architecture, nutrient acquisition, and cold stress tolerance. In *Seed Endophytes: Biology and Biotechnology*. Verma, S.K., and White, J.J.F. (eds). Cham, Switzerland: Springer International Publishing, pp. 139–170.
Mengual, C., Schoebitz, M., Azcón, R., and Roldán, A. (2014) Microbial inoculants and organic amendment improves plant establishment and soil rehabilitation under semiarid conditions. *J Environ Manage* **134:** 1–7.
Midha, S., Bansal, K., Sharma, S., Kumar, N., Patil, P.P., Chaudry, V., and Patil, P.B. (2016) Genomic resource of rice seed associated bacteria. *Front Microbiol* **6:** 1551.
Mitter, B., Pfaffenbichler, N., and Sessitsch, A. (2016) Plant–microbe partnerships in 2020. *Microb Biotechnol* **9:** 635–640.
Molina-Santiago, C., and Matilla, M.A. (2019) Chemical fertilization: a short-term solution for plant productivity? *Microb Biotechnol* 1–3.
Naylor, D., Degraaf, S., Purdom, E., and Coleman-Derr, D. (2017) Drought and host selection influence bacterial community dynamics in the grass root microbiome. *ISME J* **11:** 2691–2704.
Nguyen, N.H., Song, Z., Bates, S.T., Branco, S., Tedersoo, L., Menke, J., *et al*. (2016) FUNGuild: AN open annotation tool for parsing fungal community datasets by ecological guild. *Fungal Ecol* **20:** 241–248.
Ok-Sun, K., Yong-Joon, C., Kihyun, L., Seok-Hwan, Y., Mincheol, K., Hyunsoo, N., *et al*. (2012) Introducing EzTaxone: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int J Syst Evol Microbiol* **62:** 716–721.
Pitzschke, A. (2016) Developmental peculiarities and seed-borne endophytes in Quinoa: omnipresent, robust bacilli contribute to plant fitness. *Front Microbiol* **7:** 2.
Rojas, X., Guo, J., 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.
Rolli, E., Marasco, R., Vigani, G., Ettoumi, B., Mapelli, F., Deangelis, M.L., *et al*. (2014) Improved plant resistance to drought is promoted by the root-associated microbiome as a water stress-dependent trait. *Environ Microbiol* **17:** 316–331.
Rybakova, D., Mancinelli, R., Wikström, M., Birch-Jensen, A.S., Postma, J., Ehlers, R.U., *et al*. (2017) The structure of the Brassica napus seed microbiome is cultivar-dependent and affects the interactions of symbionts and pathogens. *Microbiome* **5:** 104.
Shahzad, R., Khan, A.L., Bilal, S., Asaf, S., and Lee, I.J. (2018) What is there in seeds? Vertically transmitted endophytic resources for sustainable improvement in plant growth. *Frontiers. Plant Sci* **9:** 24.
Shearin, Z.R.C., Filipek, M., Desai, R., Bickford, W.A., Kowalski, K.P., and Clay, K. (2018) Fungal endophytes from seeds of invasive, non-native Phragmites australis and their potential role in germination and seedling growth. *Plant Soil* **422:** 1–12.
Sheibani-Tezerji, R., Naveed, M., Jehl, M.-A., Sessitsch, A., Rattei, T., and Mitter, B. (2015) The genomes of closely related *Pantoea ananatis* maize seed endophytes having different effects on the host plant differ in secretion system genes and mobile genetic elements. *Front Microbiol* **6:** 440
Tedersoo, L., Anslan, S., Bahram, M., Põlme, S., Riit, T., Liiv, I., *et al*. (2015) Shotgun metagenomes and multiple primer pair-barcode combinations of amplicons reveal biases in metabarcoding analyses of fungi. *MycoKeys* **10:** 1–43.
Thomashow, L.S., LeTourneau, M.K., Kwak, Y.-S., and Weller, D.M. (2019) The soil-borne legacy in the age of the holobiont. *Microb Biotechnol* **12:** 51–54.
Toju, H., Peay, K.G., Yamamichi, M., Narisawa, K., Hiruma, K., Naito, K., *et al*. (2018) Core microbiomes for sustainable agroecosystems. *Nature Plants* **4:** 247–257.
Truyens, S., Weyens, N., Cuypers, A., and Vangronsveld, J. (2015) Bacterial seed endophytes: genera, vertical transmission and interaction with plants. *Environ Microbiol Rep* **7:** 40–50.
Walitang, D.I., Kim, K., Madhaiyan, M., Kim, Y.K., Kang, Y., and Sa, T. (2017) Characterizing endophytic competence and plant growth promotion of bacterial endophytes inhabiting the seed endosphere of Rice. *BMC Microbiol* **17:** 209.
Xiang, H., Zhang, Y., Wei, H., Zhang, J.-E., and Zhao, B. (2018) Soil properties and carbon and nitrogen pools in a young hillside longan orchard after the introduction of leguminous plants and residues. *PeerJ* **6:** e5536.
Yang, L., Shan, Z., Zou, Y., Wang, J., and Wei, S. (2013) Investigation on diversity and population succession dynamics of endophytic bacteria from seeds of maize (Zea mays L., Nongda108) at different growth stages. *Ann Microbiol* **63:** 71–79.
Yang, S., Zhang, X., Cao, Z., Zhao, K., Wang, S., Chen, M., and Hu, X. (2014) Growth-promoting Sphingomonas paucimobilis ZJSH1 associated with Dendrobium officinale through phytohormone production and nitrogen fixation. *Microb Biotechnol* **7:** 611–620.
Yang, L., Danzberger, J., Schöler, A., Schröder, P., Schloter, M., and Radl, V. (2017) Dominant groups of potentially active bacteria shared by barley seeds become less abundant in root associated microbiome. *Front Plant Sci* **8:** 1005.
Yumlembam, R., and Borkar, S.G. (2014) Assessment of antibacterial properties of medicinal plants having bacterial leaf endophytes against plant pathogenic Xanthomonads. *Indian Phytopathol Soc* **67:** 353–357.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Different Phylogenetic analysis of the seven forage seeds.
Fig. S2. Ring-charts showing the community structures associated with the investigated forage seeds.
Fig. S3. Significant co-occurrence and co-exclusion relationships among the fungal microbiome of forage seeds.
Fig. S4. The effects of different stains on germination rate of Medicago sativa under salt stress.
Fig. S5. The economically important forage seeds used in this study as sources of endophytes.
Table S1. Relative proportions of predicted function of 16S rRNA gene with significant difference between the Elymus and other forages.
Table S2. Relative proportions of predicted functions of plant growth-promoting (PGP) and sporulation traits.

