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Running Title: Response of endophytic bacteria to fertilization

Fertilization impacts bacterial communities in wheat endospheres

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Highlights

- Endophytic bacterial community composition dramatically differed between leaves and roots, and long-term fertilization significantly changed their community compositions.
- Many of the keystone species in the endophytic bacterial co-occurrence networks of wheat leaves and roots were involved in plant growth and fitness.
- The total relative abundance of keystone species in NPK plus cow manure treatment was highest in both leaves and roots.

ABSTRACT

Fertilization has been shown to exert significant influence on soil microorganisms, and directly and indirectly influences plant growth and survival in agroecosystems. However, it is unknown whether fertilization impacts endophytic microbial communities, which are ubiquitous and are intimately associated with plant growth and health. Here, we investigated endophytic bacterial communities in wheat leaves and roots under different long-term fertilization regimes, including NPK chemical fertilizer and NPK chemical fertilizer combined with wheat straw, pig manure, or cow manure. Endophytic bacterial community composition dramatically differed in leaves and roots. Different fertilization treatments did not affect the endophytic bacterial species richness or

phylogenetic diversity in either leaves or roots, but did significantly alter community compositions, particularly in roots. The endophytic bacterial co-occurrence network in leaves was more complex and stable than that in roots. Furthermore, many of the keystone species that we identified by their topological positions in the co-occurrence networks of leaves and roots were involved in plant growth and fitness. The total relative abundance of keystone species was highest in the NPK plus cow manure treatment in both leaves and roots. Overall, our results suggest that different fertilization managements can strongly impact endophytic bacterial communities, and the combination of NPK fertilizer and cow manure have a promotion on the relative abundance of the key endophytic bacterial microbiota in both leaves and roots, which might be beneficial for plants in agroecosystems.

Key Words: endophytic bacterial community, long-term fertilization, organic matter, key microbiota, network analysis.

INTRODUCTION

Plants can be viewed as complex systems with an intimately associated microbiome, much like the relationship between humans and our own microbiomes (Gordon, 2012). Plant-dwelling microorganisms are crucial for plant growth and health, as well as playing a fundamental role in the adaptation of the plant to diverse environments (Vandenkoornhuyse *et al.*, 2015). Plant endosphere and ectosphere of both aboveground and belowground compartments provide diverse habitats for microbial communities, and distinctive microbiota become established within each plant part (Vandenkoornhuyse *et al.*, 2015). Endophytic bacteria, which commonly occur within plants without causing disease symptoms, can be involved in major physiologic functions, such as nutrient acquisition (Matos *et al.*, 2017), pathogen defense (Verma and White, 2018) and abiotic stress tolerance (Abd Allah *et al.*, 2018). Hence these organisms may promote host plant growth and productivity in agricultural ecosystems (Rehman *et al.*, 2018). In order to effectively utilize microbiota to influence plant growth and productivity in agricultural systems, it is imperative for us to examine the driving factors controlling endophytic communities. Studies to date have explored plant-associated microbial communities associated with some crop plant species under agricultural environments. For example, the phyllosphere and rhizosphere of rice, respectively, were colonized by particular bacterial communities with physiological traits of differential importance, such as transport processes and stress responses were more conspicuous in the phyllosphere while dinitrogenase reductase was exclusively identified in the rhizosphere (Knief *et al.*, 2012). Individual microbial populations in the maize rhizosphere were strongly modified by crop genotype (Aira *et al.*, 2010). And the dominant factors influencing microbial community composition in wheat rhizosphere included both plant age and site (Donn *et al.*, 2015). However, in contrast to the well-studied rhizosphere and phyllosphere microbiome, the more intimate associations of plants with their endophytic microbiota are less well understood. Improving our knowledge of plant endophytes is critical because these organisms are likely to have a strong influence on plant physiology.

Interspecific interactions can influence the composition and development of the microbiome

and the interconnected microbiome found within plant tissues can critically affect plant physiology both directly and indirectly (van der Heijden and Hartmann, 2016). Co-occurrence networks are a helpful approach to better understand the potential direct or indirect interactions between microbial species. These networks are constructed by calculating pairwise correlations based on the relative abundance of individual taxa (van der Heijden and Hartmann, 2016). Microbial taxa that are highly connected within the network potentially exert a considerable influence on the microbiome, irrespective of their abundance, are proposed to be keystone taxa (Banerjee *et al.*, 2018). These species may have a strong and unique influence on microbial communities and, therefore, may have a greater contribution to the system overall (Banerjee *et al.*, 2018). For example, by examining the effect of the two keystone microbes (isolates of *Albugo* and *Dioszegia*) on other phyllosphere microbiota, it was found that the presence or absence of these keystone microbes could have disproportionately large effects among phyllosphere microbiota (Agler *et al.*, 2016). In the rhizosphere, a keystone species in the genus *Mesorhizobium* was responsible for production of alkaline phosphomonoesterase (ALP) and was positively correlated with the most dominant bacterivores in the nematode genus *Protorhabditis* ((Jiang *et al.*, 2017). However, interspecific interactions and potential keystone microbial species of plant endophytes remains largely unexamined.

In agroecosystems, fertilization has long been a primary focus of research as it is an extensive and efficacious management measure. Long-term fertilization is known to change the soil environment and can thereby exert a profound influence on the native microbial communities upon which crops depend (Shen *et al.*, 2010). In one study of the effects of long-term fertilization treatments, the variation in soil bacterial community composition was mainly driven by soil pH (Sun *et al.*, 2015). In addition, bacterial community composition in the maize rhizosphere has been shown to be most strongly influenced by soil pH, soil organic matter, and available phosphorus (Wang *et al.*, 2018). However, in comparison to soil and rhizosphere communities, crop endophytic populations may play a more direct role in helping a host plant adapt quickly to changing environmental conditions (Doty, 2017). Despite the abundance of research on the effects of fertilization in agroecosystems and the importance of endophytes to crop physiologic function, the responses of endophytic microbial taxa to fertilization remain unexplored. Here, we proposed that applications of long-term fertilizer treatments could have an influence on crop endophytic communities. We compared bacterial endophytic assemblages within winter wheat leaves and roots subjected to 35 years of either chemical fertilizer or fertilizer supplemented with organic matter inputs, including wheat straw, pig manure and cow manure. We also identified putative keystone species among the endophytic bacterial communities through co-occurrence network analyses. Three specific questions are addressed: (1) How do plant parts and long-term fertilization affect endophytic bacterial communities? (2) Are the patterns of interactions within endophytic bacterial communities in leaves and roots different? (3) Does fertilization affect the relative abundance of keystone species in the endophytic bacterial communities of leaves and roots?

MATERIALS AND METHODS

Study sites and experimental design

The long-term field experimental site has been in wheat-soybean crop rotation since 1982. The site is located in Mengcheng, Anhui province, China (33°13′N, 116°35′E). The soil in this region is classified as a typical lime concretion black soil. Five treatments with four replicate plots (a total of 20 plots) for each were included in this experiment: no fertilization (Control); NPK chemical fertilizers only (NPK); NPK chemical fertilizers combined with wheat straw (NPK+WS); NPK chemical fertilizers combined with pig manure (NPK+PM); and NPK chemical fertilizers combined with cow manure (NPK+CM). The NPK chemical fertilizers were composed of urea (180 kg N ha^{-1} $year^{-1}$), superphosphate (90 kg P_2O_5 ha^{-1} $year^{-1}$) and potassium chloride (135 kg K_2O ha^{-1} $year^{-1}$). Wheat straw (straw pieces about 10cm), pig manure, and cow manure additions were 7,500, 15,000 (fresh weight) and 30,000 (fresh weight) kg ha^{-1} $year^{-1}$, respectively. All fertilizers were applied once a year in October, prior to sowing of winter wheat (*Yannong 19*).

Sample collection and analysis

Wheat leaf and root samples were collected on April 21, 2017, during the booting of winter wheat. In each plot, all leaves below the flag leaf were picked from 30 randomly selected healthy wheat tillers. The root systems of plants selected for leaf collection were dug up in the field, and nearly complete roots were collected from each of these wheat tillers. All leaf and root samples from the same plot were pooled into sealed polyethylene bags for leaves and roots, respectively, and stored at 4℃ in coolers for transport. All samples were brought to the laboratory within 12 hours where each sample was divided into two subsamples. One subsample was used for the determination of plant tissue element concentration, and the other subsample was used for DNA extraction.

Concentration determinations of thirteen elements of leaves and roots in each plot were quantified to establish the nutrient status of wheat crops under fertilizer treatments (TABLE SⅠ). Plant tissue for nutrient determinations were ground into a fine powder using a clean ball grinder (DHS TL-2010S, China). Total carbon (TC) and nitrogen (TN) were measured using a C/N elemental analyzer, Vario MAX (Elementar, German). The remaining powder of each plant sample was digested in a concentrated HNO_3 and $HClO_4$ solution (4:1, v/v) for determination of total phosphorus (TP), total potassium (TK), Ca, Mg, S, and microelements, including Na, Fe, B, Mn, Zn and Cu with ICP, Optima 8000 (PerkinElmer, USA).

Surface sterilization

All plant tissues from the other subsample (leaves and roots) were surface sterilized following a modification of the ethanol-sodium hypochlorite method according to Sun *et al.* (2008). Samples were rinsed with sterile water to remove all attached soil, then soaked in 70% ethanol (2min), 2% NaOCl (5min), and 70% ethanol (30s), and finally washed with sterile deionized water five times. Excess moisture was subsequently removed with sterile filter paper. Water from the final rinse of each sample was spread on a TSA (Tryptic Soy Agar) plate and the plates were incubated in an aseptic room. As no bacteria were found on the plates after incubation we confirmed surface sterilization was effective. Thus, we referred to the bacterial communities remaining after surface sterilization in this study as endophytic bacterial communities. Following

sterilization, each sample was ground and homogenized with liquid nitrogen using sterile mortars and pestles in a sterile room, placed in airtight aseptic tubes, and stored at -40℃ until further processing.

DNA extraction and amplicon sequencing

Total DNA was extracted from the surface-sterilized sample powder (0.10g) using DNeasy Plant Mini Kit (Qiagen, Germany), with a modified standard extraction protocol as described by Zimmerman and Vitousek (2012). Briefly, the recommended amounts of buffer AP1 and P3 were doubled, ten 2.3mm Zirconia/Silica beads were used to improve vortexing, and the incubation step was extended to 60min. DNA concentrations were quantified using a NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). The hypervariable regions V5-V7 were amplified in triplicate from the DNA extracts using the bacterial primers 799F (5′-AACMGGATTAGATACCCKG-3′) and 1193R (5′-ACGTCATCCCCACCTTCC-3′) (Bulgarelli *et al.*, 2015). In brief, PCRs were performed with the TransStart Fastpfu DNA Polymerase (AP221-02, TransGen, China) system in a total volume of 20μL under the following reaction: 3μL template DNA (10 ng/μL), 4μL FastPfu buffer, 2μL 2.5mM dNTPs, 0.8μL each of 5μM forward and reverse primers, 0.4μL FastPfu polymerase and 0.2μL BSA; temperature cycling: 95℃ for 3min, 13 cycles of 95℃ for 30s, 55℃ for 30s and 72℃ for 45s, then 72℃ for 10min, 4℃ until use. The resulting PCR products were run on a 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), then subsequently quantified using QuantiFluor™-ST (Promega, USA) according to the manufacturer's protocol. The purified amplicons were pooled in equimolar concentrations for paired-end sequencing (2 × 250) on an Illumina MiSeq platform according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The raw data was submitted to the Sequence Read Archive (SRA) at NCBI with accession number SRP143450.

Data processing and statistical analysis

Raw data were subjected to quality filtering (Q>30), length controlled (>380bp), chimera checking and OTU clustering using the QIIME pipeline (http://qiime.sourceforge.net/) (Caporaso *et al.*, 2010). In brief, chimeras were identified using UCHIME and the ''gold'' reference database (http://drive5.com/uchime/gold.fa). Operational taxonomic units (OTUs) were clustered with 97% similarity threshold using the uclust method with default parameters (*pick_otus.by* script) (Edgar, 2010). Phylogenetic trees were constructed with FastTree after aligning representative sequences with PyNast (http://qiime.org/pynast/). The taxonomic identity of each OTU was assigned by uclust algorithm against the SILVA (v128) database (Quast *et al.*, 2013). In total, 291,631 high-quality sequences across 40 samples were clustered into 3,127 OTUs after further removing singletons and unassigned taxa (*filter_taxa_from_otu_table.py* script). Because the minimum sequence depth in the samples was 4,767, then all samples were resampled at a sequence depth of 4,700 (*single_rarefaction.py* script) with 2,994 OTUs obtained. Two alpha diversity metrics, observed_species (Species richness) and PD_whole_tree (Phylogenetic diversity), were calculated by the command *alpha_diversity.py* rarefied at 4,700 counts per sample.

Differences in plant traits and alpha diversity were tested to determine significance with the

Independent t-test and ANOVA analysis (TukeyHSD) in SPSS 20.0 for Windows. To examine patterns in beta diversity, principal coordinate analyses (PCoA) were conducted on the Bray-Curtis dissimilarity in R package vegan (https://CRAN.R-project.org/package=vegan). Significant differences in community composition were carried out using analysis of similarities (ANOSIM) in the vegan package. Mantel tests were performed to examine the significant correlation between endophytic bacterial communities and plant traits in leaves and roots, respectively. Figures were generated in R with package ggplot2 (Ginestet, 2011).

The construction of co-occurrence networks used Spearman correlation coefficients with the "WGCNA" package (Langfelder and Horvath, 2012), and the properties of these networks were calculated with the "igraph" package in R (http://igraph.org). Any OTUs with relative abundances less than 0.01% were removed, and *P* values were corrected (adjusted $P<0.001$) for multiple testing through the Benjamini and Hochberg (FDR) method (Benjamini *et al.*, 2006) in "multtest" package (http://www.bioconductor.org/packages/release/bioc/html/multtest.html). The network of significant correlations ($\rho>0.6$, $P<0.001$) were visualized in Gephi (http://gephi.github.io/). Putative keystone taxa were identified statistically through microbial network analyses. Each node was assigned a role according to its topological properties (z and c values) (Guimera and Amaral, 2005). Four roles were characterized, namely: peripherals ($z \leq 2.5$ and $c \leq 0.62$), module hubs ($z \geq 2.5$ and $c \leq 0.62$), network hubs ($z \geq 2.5$ and $c \geq 0.62$) and connectors ($z \leq 2.5$ and $c \geq 0.62$). Peripheral species have few links with others in the microbial network; module hubs have only high links inside their own modules (a high z value) and are important to their own module coherence; network hubs have both high z and c values and thus are taken to have importance to the coherence of both their own module and the whole network; and connectors which have only a high c value are critical to maintain network coherence (Poudel *et al.*, 2016). Because they have many connections in the network, OTU identified as module hubs, network hubs, and connectors are referred to here as putative keystone taxa.

RESULTS

Dominant endophytic bacterial taxa

In total, 2,994 OTUs were obtained after resampled at a sequence depth of 4,700. Among them, the OTU richness in roots (2,713) was substantially greater than that in leaves (700), with an overlap of 419 OTUs (Fig. S1). Only 2.2% of OTUs could be classified at the species level. The top five phyla in the endophytic bacterial community were Actinobacteria (43.5%), Proteobacteria (33.3%), Bacteroidetes (13.5%), Firmicutes (7.4%) and Fusobacteria (1.9%). The endophytic bacterial communities in leaves were strongly dominated by Actinobacteria, which accounted for 70.6% of the sequences, whereas the communities in roots were dominated by Proteobacteria (49.1%) (Fig. 1).

At the genus level, the top five genera identified in the leaf endophytic bacterial community were *Nocardia* (66.5%), *Achromobacter* (6.6%), *Sneathia* (3.4%), *Bacillus* (1.5%) and *Pseudomonas* (1.4%). Fertilization management did not change the relative abundance of these genera compared to the control treatment (Fig. S2). In the root endophytic bacterial community, *Flavobacterium* (20.2%), *Pseudomonas* (19.9%), *Bacillus* (8.6%), *Rhodococcus* (6.3%) and

Janthinobacterium (4.4%) were the most abundant genera. In comparison to the control, the relative abundance of *Flavobacterium* was significantly higher in the NPK + pig manure treatment (Fig. S3). The genera *Pseudomonas*, *Bacillus*, *Rhodococcus* and *Janthinobacterium* had no significant change in their relative abundances. Furthermore, we regarded all the OTUs with relative abundance over 1.0% together as the abundant microbiota, which accounted for 78.5% and 54.5% of the total community in leaves and roots, respectively. We found that fertilizer management did not have significant influence on the total relative abundance of the abundant microbiota as a proportion of the total community in either leaves or roots (Fig. S4).

Endophytic bacterial diversity and community composition

The alpha diversity of bacterial endophytes was much higher in roots than in leaves; but fertilization treatment did not significantly affect the species richness and phylogenetic diversity in either leaves or roots as compared to the control (Fig. S5). However, while PERMANOVA revealed that plant parts contributed to the largest proportion of variation in beta diversity of endophytic bacterial community composition (42% of variance, P=0.0001, TABLE Ⅰ), fertilizer treatment contributed to 12% of this variation (P=0.0019). And for each plant part considered individually, endophytic bacterial community composition was influenced by fertilizer treatment (Fig. 2). Meanwhile, ADONIS analyses showed the explained variances of fertilization to the bacterial community structure in roots and leaves were 47.1% (P=0.001), 31.3% (P=0.008), respectively. ANOSIM analyses revealed that all the fertilization treatments caused significant differences in endophytic communities structure as compared with the control; and there were greater impacts on the community in roots (R=0.75, P=0.001) than that in leaves (R=0.42, P=0.001) (TABLE SⅡ). Also, the communities in roots were affected by all treatments with the supplementation of organic matter compared to NPK alone; while in leaves, significant differences existed between the addition of livestock manure (pig or cow manure) and NPK treatment but not between supplemental wheat straw addition and NPK treatment. Mantel tests showed the variation in the leaf endophytic bacterial community was primarily driven by TC, whereas TP, TN, Ca, Na and Zn were each significantly correlated with the root endophytic bacterial community (TABLE SⅢ).

Associations within endophytic bacterial communities of wheat leaves and roots

Co-occurrence networks of endophytic bacterial communities contained OTUs extensively belonging to Proteobacteria in both leaves (46.3% of taxa included in network) and roots (50.3% of taxa included in network) (Fig. 3A). As a whole, the majority of correlations between OTUs were positive in both leaves (99.8%) and roots (81.2%). However, there were strong differences in endophytic bacterial co-occurrence patterns between leaves and roots (TABLE SⅣ). Although microbial network had more bacterial members in roots than in leaves (491 vs. 285 nodes), more correlations between the members was found in leaves than in roots (2567 vs. 2466 edges), suggesting the endophytic bacterial members were more connected to each other in leaves than in roots (average node degree of 18.0 vs. 10.0; TABLE SⅣ). Moreover, the endophytic bacterial network in leaves had greater connectedness among members (clustering coefficient of 0.7 vs. 0.3), interacting bacterial consortia (modularity of 0.6 vs. 0.5) and network complexity

(Connectance of 0.03 vs. 0.01; TABLE SⅣ). The distribution of the number of interactions for individual OTUs followed a power-law function in both leaves (P=0.024) and roots (P=2.5E-08), indicating a non-random distribution of OTU interactions and a hub-based structure for each of these co-occurrence networks (Fig. 3B). In other words, within each network there were consortia of OTU which interacted with each other, with fewer OTU interacting with OTU outside of their own consortium. In addition, we found the natural connectivity of the network in the leaf was greater than that of the root, suggesting the leaf network was more robust (Fig. 3C).

Keystone species in wheat leaves and roots

We used co-occurrence networks to explore the potential keystone species in endophytic bacterial communities of wheat leaves or roots. Namely, the co-occurrence network position of each OTU within both its own consortium (module) and with regard to other consortia (modules) was measured by zc-scores (Fig. 4). OTU that had few interactions (i.e. peripheral nodes) dominated both networks (97.2% and 82.3% in leaf and root networks, respectively), with OTU that may have had few interactions but belonged to two or more consortia (i.e. connectors) the only other species role (2.8%) in the leaf network and the second largest role (15.5%) in the root network (Fig. 4). Potential keystone species were defined as OTU that had a large number of interactions with other OTU in their own consortia (i.e. module hubs), a large number of interactions overall (network hubs), or OTU that played a role in connecting multiple consortia to each other (i.e. connectors). There were 8 potential keystone taxa in wheat leaves and 87 in roots. All keystone OTU were assigned a taxonomic name at the species level based on NCBI blast (TABLE SⅤ). Together, the keystone taxa accounted for 1.1% and 16.5% relative abundance in leaves and roots, respectively. This suggests that some low abundance species may act as keystone taxa, potentially having larger effects on community structure and interactions than apparent based on their proportional abundance alone.

On the whole, there were a large proportion of keystone species in both leaves and roots belonging to the class Flavobacteriia. In addition, root communities had a large proportion of keystone species in the Betaproteobacteria, Alphaproteobacteria, Actinobacteria, Bacilli and Gammaproteobacteria (Fig. 5A). The composition of keystone species at the genus level is displayed in TABLE SⅥ. The variation of the key microbiota (collection of putative keystone species in each respective network) based upon their relative abundance among different fertilizer treatments was examined. In comparison to the control, the relative abundance of the key microbiota were significantly lower in NPK fertilization in both leaves and roots. Supplementation with organic matter (wheat straw, pig manure or cow manure) significantly increased the relative abundance of the key microbiota in roots compare with NPK alone, whereas only the addition of cow manure had a significant effect in leaves (Fig. 5B).

DISCUSSION

Effects of plant tissue and fertilization on endophytic bacterial communities

Changes in the soil microbiome caused by fertilizer inputs can lead to subsequent changes in plant endosphere populations (Seghers *et al.*, 2004). In a previous study within the same

experimental field, fertilizer treatments impacted the soil bacterial community structure (Sun *et al.*, 2015). In this study, we found that fertilizer treatment also affected endophytic bacterial communities in both leaves and roots. This may be related to the shift of soil bacterial population according to the Seghers's hypothesis that changes in plant endosphere populations result from fertilizer inputs may be owing to changes in the soil microbiota. In contrast, Robinson *et al.* (2016) proposed that endophytic bacterial assemblages might be altered by fertilization through changes in recruitment caused by plant growth and/or exudates. Here, we found that the endophytic community in leaves was only significantly influenced by total C. Similarly, Yang *et al.* (2016) also observed a strong correlation between fungal endophytic communities and foliar carbon content in a natural ecosystem, indicating a potential widespread effect of foliar carbon source on endophytic microbial communities. Meanwhile, the community in roots was significantly correlated with several elements, including total P, total N, Ca, Na, and Zn, but the strength of each of these correlations was low (TABLE SⅢ). These results are in agreement with previous work that has shown only weak relationships between plant nutrient status and microbial community composition (Hamonts *et al.*, 2018). Therefore, other factors such as the quantity and chemistry of plant exudates and secondary metabolites should be considered to explain the responses of plant endophytes recruitment to fertilization.

The lack of an effect of fertilization management on endophytic bacterial alpha diversity may be a result of the strong influence of the host plant on endophytic taxa. In this study, we did not observe significant differences in endophytic bacterial alpha diversity among fertilization treatments (Fig. S5). Similarly, Seghers *et al.* (2004) found that the OTU richness of the maize root endophytic microbiome did not differ between conventional and organic management. And in a comparison among different root compartments, Edwards *et al.* (2015) found an effect of conventional and organic cropping management on bacterial diversity in the rhizosphere, but not in the rhizoplane or the endosphere. It seems that the more intimately linked the bacterial microbiota is to the host plant, the less its diversity is affected by soil management strategy.

Hub-based co-occurrence networks in leaves and roots

Co-occurrence networks were built to examine the interconnections among bacterial populations within the plant leaf and root endospheres. Patterns in the frequency, strength, and distribution of interactions among OTU in the endophytic communities of leaves and roots varied (network topological properties in TABLE SⅣ). Although the size of the microbial network in leaves was smaller than that in roots, the network in leaves had more connections among taxa and higher correlations for per bacterial member with other members, indicating closer associations among the endophytic taxa in leaves. Moreover, there was stronger clustering into and within consortia in the leaf endophytic network versus that in roots. This stronger clustering may reflect the importance of ecological processes, such as degradation pathways that involve cooperation among multiple taxa, in shaping the leaf endophytic bacterial community (Röttjers and Faust, 2018). Considered together, these factors suggest a more complex set of interactions influencing the structure of the endophytic bacterial community in leaves than in roots.

These differences in interactions among OTU within a community may be a result of niche

differentiation between these plant parts (Muller *et al.*, 2016). Overall, positive associations were common in both leaves and roots (Fig. 3A). We found more negative correlations in the root endosphere network compared to the leaf endosphere network (TABLE SⅣ), which is possibly attributable to the variance in carbon resource availability among these plant parts. Leaves contained more carbon than roots (TABLE SⅠ) and there was less evidence of competition (lower proportion of negative correlations) in the microbial network of leaf endosphere, indicating that greater resource availability may decrease competition in microbial communities (Costello *et al.*, 2012).

We also found that both endophytic bacterial co-occurrence networks showed a hub-based structure with the distribution of connections among individual OTU, following a power-law distribution (Bergman and Siegal, 2003) (Fig. 3B). While the distribution of the OTU interactions in leaves and roots both followed a power law function, but it showed there were fewer OTU with many connections to other OTU in the endophytic community of leaves than that in roots (Fig. 3B). Because there were fewer OTU with many connections in the leaf community, these OTU are (statistically) less likely to be lost at random; thus, the leaf network may be more stable to non-taxon specific perturbations (Röttjers and Faust, 2018). In addition, the network in leaves had higher clustering coefficient, modularity, connectance and natural connectivity (TABLE SⅣ and Fig. 3C), suggesting that the endophytic bacterial network had a higher complexity with stronger connectivity in leaves than in roots. Complex networks with greater connectivity have been found to be more robust to environmental perturbations (Santolini and Barabasi, 2018). In this sense, the leaf microbiota in this study might be more resilient to environmental stresses as different taxa or functions can complement each other.

Predicted functions and importance of key microbiota in wheat leaves and roots

We considered the traits of these putative keystone species to ascertain whether they might possess traits impacting the stability of microbial interaction networks or otherwise be crucial for plant growth and health (Agler *et al.*, 2016). Taken together, the most dominant genus of the key microbiota in both leaves and roots belonged to the genus *Flavobacterium*, which also had the highest number of connections to other taxa (TABLE SⅥ). This genus has been found to be an important member of the root- and leaf-associated microbiome in multiple studies, and has been found to have beneficial functions supporting plant growth and resistance to pathogens (Manter *et al.*, 2010; Kolton *et al.*, 2014). In addition, some genera in the key microbiota had a large number of interactions despite having relative abundances below 0.10% and are known to have effects on plant growth or health (TABLE SⅥ). For example, in the leaf key microbiota, the genera *Chryseobacterium* (0.05%, degree=23) and *Pseudomonas* (0.01%, degree=14) were previously reported as plant growth promoting rhizobacteria (PGPR) (Singh *et al.*, 2013; Dorjey *et al.*, 2017). In the root key microbiota, the genera *Mesorhizobium* (0.08%, degree=46) and *Paenibacillus* (0.04%, degree=43) are known to have many species which act as nitrogen-fixing bacteria (Moscatiello *et al.*, 2015; Grady *et al.*, 2016). Moreover, other genera detected in the key microbiota of leaves or roots are known to possess functional traits to promote plant fitness, including *Bacillus*, *Rhizobium*, *Paraburkholderia*, *Thiobacillus*, *Sphingomonas*, *Mycobacterium*,

Microbacterium, *Rhodococcus* and *Bradyrhizobium* (Trivedi *et al.*, 2007; Ansori and Gholami, 2015; Khan *et al.*, 2017; Lemanceau *et al.*, 2017; Cordovez *et al.*, 2018). Thus we propose that the remaining keystone taxa we identified may also be important for host plants; although the influence of these taxa which have not previously been recognized as plant beneficial taxa needs further study and confirmation.

The less abundant key microbiota were affected by different fertilization managements

Many studies have focused on differences in the abundant taxa of a community, because these taxa have been shown acting as a main driver for community patterns (Shade and Handelsman, 2012). However, in ecological systems most species are rare, and the importance of rare members have been highlighted as these taxa often have distinct functional traits from the abundant species in many studies (Mouillot *et al.*, 2013; Shade *et al.*, 2014). The influence of keystone taxa, rare community members with a disproportionate influence on community stability or functioning, also emphasize the potential importance of numerically inconspicuous taxa (Banerjee *et al.*, 2018). Most of the potential keystone species identified in our study based on their position within interaction networks had low relative abundance (TABLE SⅤ). Further, the presence or loss of keystone species in certain environments may affect not only the stability of interactions within the community, but also the fitness of the host (Agler *et al.*, 2016; van der Heijden and Hartmann, 2016; Banerjee *et al.*, 2018). In our study, we found long-term fertilization affected the relative abundance of key microbiota, but not the abundant microbiota, in both leaves and roots (Fig. 5B; Fig. S5). This indicates that the effects of fertilization on whole endophytic communities may be mediated by effects on keystone taxa. Compared to the other fertilizer treatments, the supplement of cow manure significantly increased the relative abundance of keystone microbial species in both leaves and roots. This suggests that NPK fertilization combined with cow manure may be a balanced fertilization approach from the perspective of maintenance of endophytic bacterial co-occurrence networks and the accommodation of a larger proportion of keystone species. However, the actual effect of keystone species on plant growth and health still needs to be validated through well-designed experiments. Furthermore, considering the limitation of 16S rRNA gene taxonomic resolution at the species level (Větrovský and Baldrian, 2013), a more accurate phylogenetic marker such as gyrB should be considered in the validation of the responses of keystone microbes to fertilization at the species level.

In this study, we showed that fertilizer treatments can have significant influences on endophytic bacterial community composition, indicating we should take these special communities into consideration for the assessment of fertilization managements and regulation of microbial resources in agroecosystems. Furthermore, previous studies have shown that endophytic microbial communities vary among different plant genotypes, development stages, as well as seasons and years (van Overbeek and van Elsas, 2008; Marques *et al.*, 2015; Campisano *et al.*, 2017). Therefore, the response of endophytic associations to fertilization may need to be considered in light of these factors. Examining the interactions of fertilization with these other factors is necessary to understand its effects on endophytic bacterial communities. Our work provides a first step towards consideration of endophytic bacterial communities in the

development of agricultural fertilizer management strategies.

CONCLUSIONS

The endophytic bacterial communities in wheat leaves and roots were dominated by Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, and Proteobacteria. Plant part and fertilization treatment both affected bacterial community structures in the plant endosphere. The alpha diversity of bacterial endophytes in roots was much higher than that in leaves, while fertilization had no significant effect on the alpha diversity of the endophytic bacterial community. The endophytic bacterial co-occurrence network in leaves had a smaller network size (285 OTU), but a greater number of positive correlations (99.8%), and a more robust, complex structure compared with that of roots, with leaves having a higher average number of connections per OTU (node degree 18.0), and stronger clustering into and within consortia (modularity 0.6 and connectance 0.03). We identified 95 unique keystone species in the networks of wheat leaves and roots which may serve as a list of potential plant beneficial endophytic bacteria and, if so, may provide novel targets for the sustainable management of wheat crops in agroecosystems (van der Heijden and Hartmann, 2016). In a previous study, we found that the long-term fertilization of NPK plus cow manure could not only improve crop production, but also maintain bacterial diversity in soils (Sun *et al.*, 2015). In this study, we also found that this treatment (NPK + cow manure) could increase the relative abundance of the putative keystone bacterial microbiota, which are potentially beneficial for plant growth and fitness. Therefore, we suggest that the combination of chemical fertilizer and cow manure may be a good agricultural practice from the viewpoint of both below- and above-ground microbial communities.

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TABLE I

Results of PERMANOVA testing of the effects of plant part and fertilization treatment on endophytic bacterial communities

	pseudo-F	R^2	*P*
Plant part	37.84	0.42	0.0001
Fertilization	2.59	0.12	0.0019
Plant part*Fertilization	2.77	0.12	0.0019

Figure legends

Fig. 1 The relative abundance of the dominant endophytic bacterial phyla in wheat leaves and roots across fertilization treatments. Phyla with a total relative abundance of < 1% are grouped in "Others".

Fig. 2 Variation of endophytic bacterial communities between samples in principal coordinate analyses (PCoA) of Bray-Curtis distances in leaves and roots, respectively.

Fig. 3 Co-occurrence network patterns of winter wheat leaves and roots. (A) Co-occurrence networks visualizing significant associations ($\rho>0.6$, $P<0.001$) between endophytic bacterial OTUs in leaves and roots, respectively. Each dot represents an endophytic bacterial OTU, color represents different phyla, and node size represents degree. Edges denote significant relationships between OTUs. (B) Node degree distribution of the co-occurrence networks in wheat leaves and roots. A power-law curve is fitted to plots (shown in red). (C) Robustness of the co-occurrence networks in wheat leaves and roots.

Fig. 4 Distribution of network roles by analyzing module features in the networks of wheat leaves and roots, respectively.

Fig. 5 Taxonomic composition of keystone species in leaves and roots is reported as proportional OTUs counts per class (A). The relative abundance of the key microbiota (all keystone species) in wheat leaves and roots responded to different fertilization managements, respectively (B).

Fig. 1

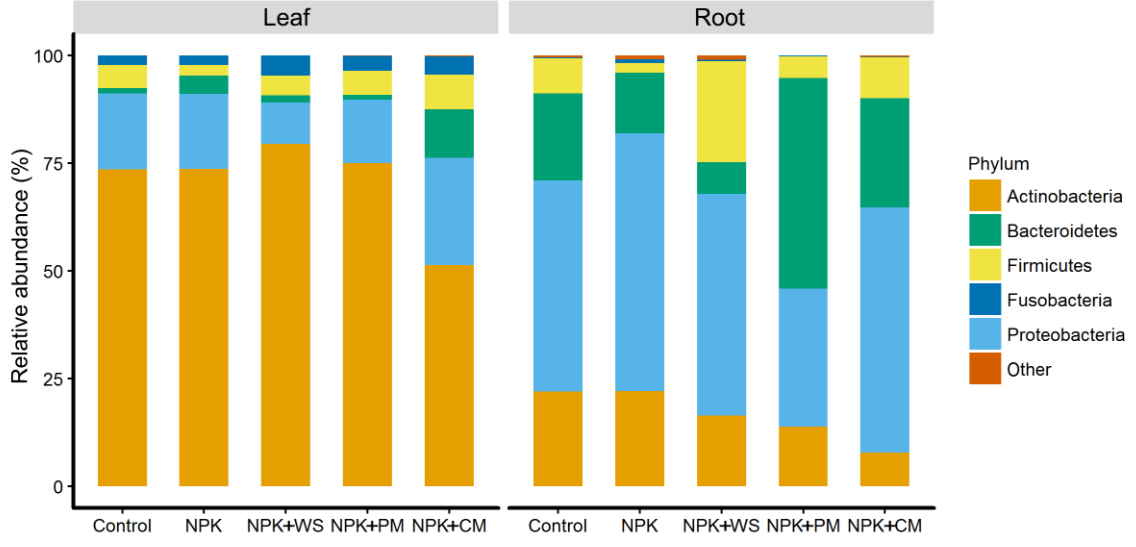


Fig. 2

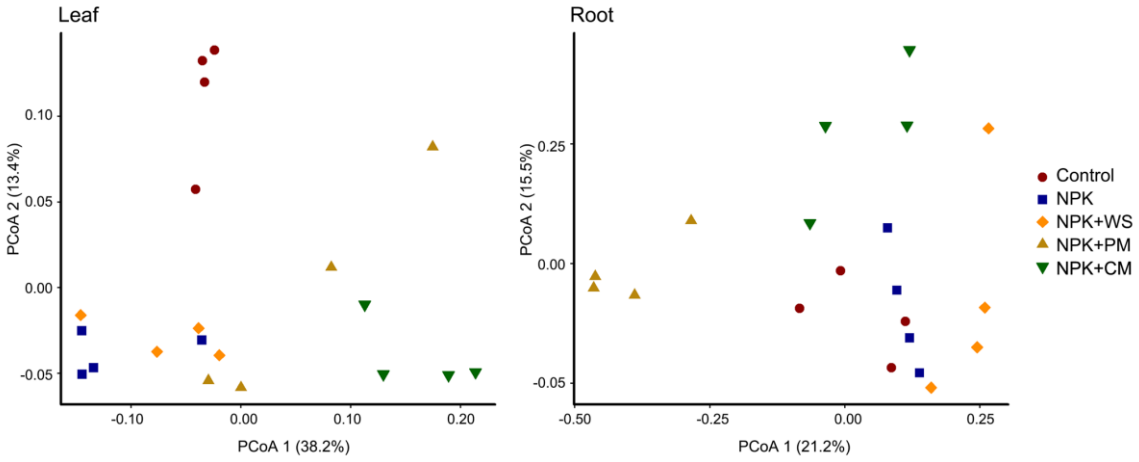

Leaf
Root
PCoA 2 (13.4%)
PCoA 1 (38.2%)
PCoA 2 (15.5%)
PCoA 1 (21.2%)
Control
NPK
NPK+WS
NPK+PM
NPK+CM

Fig. 3

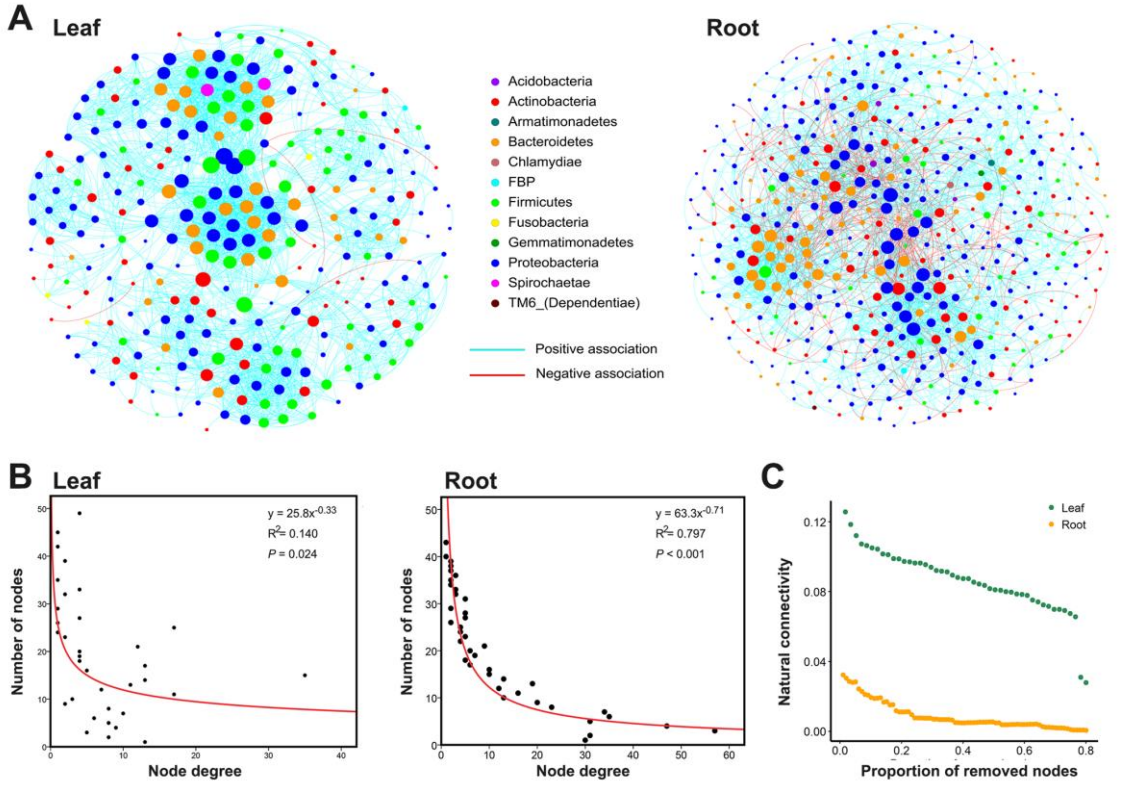

A
Leaf
Root
Acidobacteria
Actinobacteria
Armatimonadetes
Bacteroidetes
Chlamydiae
FBP
Firmicutes
Fusobacteria
Gemmatimonadetes
Proteobacteria
Spirochaetae
TM6_(Dependentiae)
Positive association
Negative association
B
Leaf
y = 25.8x-0.33
R2= 0.140
P = 0.024
Number of nodes
Node degree
Root
y = 63.3x-0.71
R2= 0.797
P < 0.001
Number of nodes
Node degree
C
Leaf
Root
Natural connectivity
Proportion of removed nodes

Fig. 4

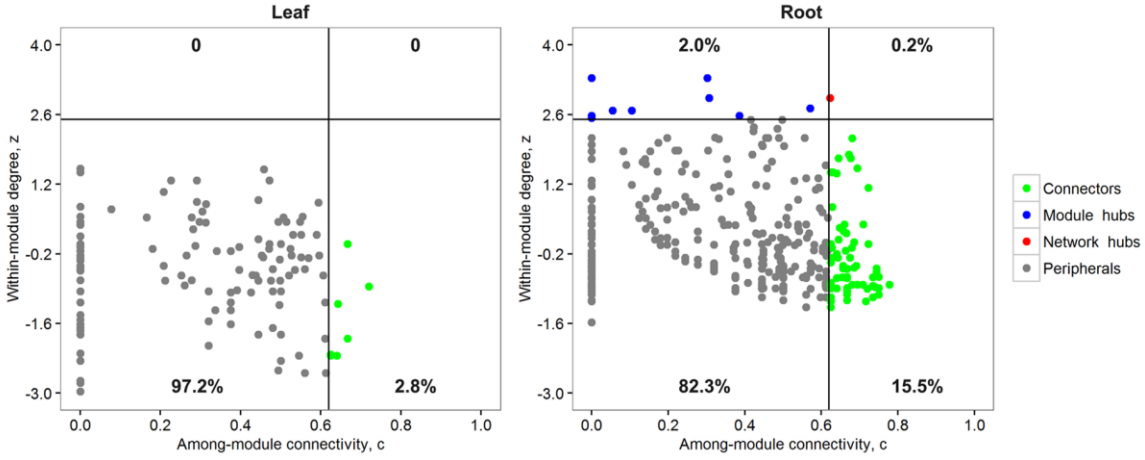
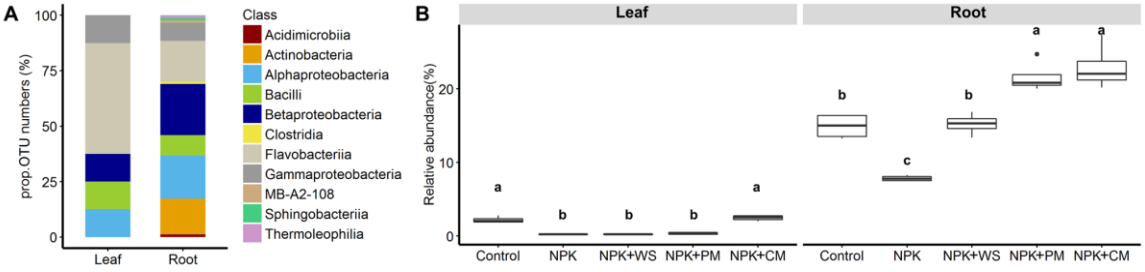

Leaf
Root
0
0
2.0%
0.2%
97.2%
2.8%
82.3%
15.5%
Within-module degree, z
Among-module connectivity, c
Connectors
Module hubs
Network hubs
Peripherals

Fig. 5


A
prop.OTU numbers (%)
Leaf
Root
Class
Acidimicrobiia
Actinobacteria
Alphaproteobacteria
Bacilli
Betaproteobacteria
Clostridia
Flavobacteriia
Gammaproteobacteria
MB-A2-108
Sphingobacteriia
Thermoleophilia
B
Relative abundance(%)
Control
NPK
NPK+WS
NPK+PM
NPK+CM

Supplementary Material

Fertilization impacts bacterial communities in wheat endospheres

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TABLE S Ⅰ

Nutrient elements in winter wheat leaves and roots under different fertilization managements. Mean values (SE) reported by the different letters in each column denote significant difference between treatments detected by Turkey pairwise comparisons at $P < 0.05$; NPK: NPK fertilizer only; NPK+WS: NPK fertilizer plus wheat straw; NPK+PM: NPK fertilizer plus pig manure; NPK+CM: NPK fertilizer plus cow manure; TC: total carbon, TN: total nitrogen, TP: total phosphorus, TK: total potassium.

Plant part	Nutrient elements	Control	NPK	NPK+WS	NPK+PM	NPK+CM
Leaf	TC (g kg^{-1})	420(3)b	432(4)a	428(3)a	429(2)a	420(4)b
	TN (g kg^{-1})	21.5(1.6)c	29.5(6.5)b	30.6(2.7)b	37.1(1.4)a	36.3(4.1)a
	TP (g kg^{-1})	0.774(0.042)c	2.83(1.21)ab	2.67(0.36)b	3.90(1.02)a	3.81(0.20)ab
	TK (g kg^{-1})	15.1(2.3)c	21.1(3.0)b	25.2(2.3)a	25.6(1.5)a	25.3(0.6)a
	Ca (g kg^{-1})	4.15(0.51)a	4.39(0.40)a	3.44(0.29)b	4.39(0.22)a	4.11(0.34)a
	Mg (g kg^{-1})	1.42(0.11)bc	1.66(0.29)ab	1.35(0.13)c	1.79(0.13)a	1.59(0.12)abc
	S (g kg^{-1})	2.29(0.19)b	2.85(0.41)a	2.88(0.33)a	2.91(0.16)a	3.05(0.35)a
	Na (g kg^{-1})	0.514(0.030)a	0.463(0.149)a	0.423(0.025)a	0.464(0.063)a	0.446(0.032)a
	Fe (g kg^{-1})	0.240(0.103)a	0.195(0.040)a	0.223(0.017)a	0.170(0.017)a	0.168(0.017)a
	B (mg kg^{-1})	22.5(2.9)a	11.3(6.3)c	17.5(2.9)ab	18.6(2.5)ab	15.0(0.0)bc
	Mn (mg kg^{-1})	27.5(2.9)c	73.6(27.8)b	115.0(22.7)a	46.3(29.3)c	25.0(4.1)c
	Zn (mg kg^{-1})	13.8(2.5)ab	15.0(7.1)ab	12.5(2.9)ab	20.00(7.1)a	8.8(2.5)b
	Cu (mg kg^{-1})	12.5(5.0)a	15.0(4.1)a	16.3(2.5)a	16.3(2.5)a	13.8(2.5)a
Root	TC (g kg^{-1})	402(7)a	380(6)b	382(8)b	376(20)b	386(11)ab
	TN (g kg^{-1})	6.63(0.76)ab	5.13(1.89)ab	4.66(0.63)b	7.18(1.19)a	6.98(1.76)a
	TP (g kg^{-1})	0.366(0.053)d	0.565(0.038)c	0.579(0.042)c	1.44(0.09)a	1.04(0.07)b
	TK (g kg^{-1})	4.32(1.03)b	4.45(1.02)b	5.79(0.71)a	7.04(0.67)a	6.59(0.29)a
	Ca (g kg^{-1})	2.05(0.11)b	1.99(0.20)b	1.74(0.16)b	2.60(0.24)a	2.58(0.34)a
	Mg (g kg^{-1})	0.847(0.135)b	1.02(0.11)ab	0.913(0.090)b	1.21(0.16)a	1.00(0.17)ab
	S (g kg^{-1})	1.06(0.17)abc	0.881(0.108)c	1.00(0.11)bc	1.28(0.20)a	1.14(0.15)ab
	Na (g kg^{-1})	0.951(0.108)b	0.886(0.097)b	0.783(0.202)b	1.40(0.23)a	1.00(0.13)b
	Fe (g kg^{-1})	2.56(0.66)c	3.79(0.48)ab	3.18(0.22)abc	4.11(1.04)a	2.85(0.89)bc
	B (mg kg^{-1})	35.0(7.1)a	10.0(4.1)c	11.3(2.5)bc	17.5(2.89)b	10.0(4.1)c
	Mn (mg kg^{-1})	67.5(10.4)b	150.0(34.9)a	146.3(12.5)a	125.0(50.5)a	67.5(11.9)b
	Zn (mg kg^{-1})	25.0(4.1)b	16.3(2.5)c	15.0(0.0)c	51.3(10.3)a	13.8(2.5)c
	Cu (mg kg^{-1})	26.3(13.2)a	5.00(0.00)c	16.3(4.8)ab	20.0(0.0)ab	8.75(4.79)b

TABLE S Ⅱ

ANOSIM test of the effects of treatments on endophytic bacterial Bray-Curtis distance matrix in leaves and roots. For abbreviations, see TABLE S Ⅰ.

ANOSIM	Leaf		Root	
	R	*P*	R	*P*
All	0.42	0.001	0.75	0.001
Control vs. NPK	0.89	0.033	0.69	0.037
Control vs. NPK+WS	0.67	0.033	0.57	0.031
Control vs. NPK+PM	0.32	0.033	0.97	0.030
Control vs. NPK+CM	0.74	0.020	0.75	0.026
NPK vs. NPK+WS	0.08	0.309	0.44	0.034

NPK vs. NPK+PM	0.32	0.032	0.89	0.028
NPK vs. NPK+CM	0.78	0.028	0.72	0.041
NPK+WS vs. NPK+PM	0.14	0.131	0.95	0.033
NPK+WS vs. NPK+CM	0.65	0.036	0.58	0.063
NPK+PM vs. NPK+CM	0.11	0.253	0.82	0.033

TABLE SⅢ

Correlation coefficients (r) and significance (*p*) were determined by Mantel test: comparing differences between samples in endophytic bacterial community composition to difference between samples in nutrient elements of winter wheat leaves and roots. For abbreviations, see TABLE SⅠ.

Variables	Leaf		Root	
	r	*p*	r	*p*
TC	0.36	0.018	0.02	0.365
TN	-0.06	0.653	0.18	0.018
TP	0.01	0.423	0.38	0.001
TK	-0.09	0.671	0.09	0.141
Ca	-0.01	0.790	0.18	0.017
Mg	-0.07	0.721	0.04	0.287
S	0.08	0.243	-0.01	0.547
Na	-0.15	0.858	0.27	0.003
Fe	-0.16	0.923	0.07	0.177
B	-0.06	0.562	-0.03	0.615
Mn	-0.06	0.576	0.12	0.062
Zn	-0.03	0.409	0.26	0.002
Cu	-0.20	0.891	0.10	0.152

TABLE SⅣ

The key topological features of co-occurrence networks.

Key traits of networks	Leaf	Root	Clarifications
Node	285	491	A taxa, e.g. the bacterial OTUs in this study
Edge	2567	2466	The lines connecting nodes, representing the correlations between the OTUs
Degree	/	/	The number of direct correlations to a node in the network, representing potential correlations between a bacterial species with others
Average node degree	18.01	10.04	The average number of correlations within OTUs in the network, representing the extent to the members closely related to each other in the network
Clustering coefficient	0.711	0.338	Transitivity. The connectedness among members of a network
Modularity	0.591	0.499	The extent to forming smaller components, e.g. individual bacterial consortia in the network
Connectance	0.032	0.010	The extent to network complexity
Positive correlations	99.81%	81.22%	The relations of cooperation between the bacterial OTUs in this study
Negative correlations	0.19%	18.78%	The relations of competition between the bacterial OTUs in this study

TABLE SⅤ

Compositions of the endophytic key microbiota (Connectors and Module hubs) in the leaf and root co-occurrence networks.

Plant organ	**OTU ID**	**Relative abundance**	**Degree**	**Species role**	**NCBI Blast**	**Blast Identity**
Leaf	GQ102686.1.1348	0.88%	4	Connectors	*Acidovorax caeni strain R-24608*	98.08%
Leaf	FJ984603.1.1375	0.02%	3	Connectors	*Bacillus aryabhattai B8W22*	99.16%
Leaf	GQ092889.1.1345	0.02%	14	Connectors	*Chryseobacterium strain JS6-6*	98.48%
Leaf	GARE01155857.2.1248	0.02%	9	Connectors	*Chryseobacterium haifense strain H38*	99.07%
Leaf	KC762313.1.1430	0.04%	4	Connectors	*Flavobacterium oryzae strain Jyi-05*	96.49%
Leaf	HE574373.1.1890	0.08%	20	Connectors	*Flavobacterium aquidurense strain WB 1.1-56*	95.92%
Leaf	GU321116.1.1514	0.01%	14	Connectors	*Pseudomonas chlororaphis subsp. aurantiaca strain NCIB 10068*	97.52%
Leaf	KU515263.1.1446	0.03%	5	Connectors	*Tardiphaga robiniae strain R-45977*	97.61%
Root	JQ712558.1.1326	0.01%	27	Module_hubs	*Acidovorax valerianellae strain CFBP 4730*	95.74%
Root	GU134931.1.1453	0.19%	5	Connectors	*Actimicrobium antarcticum strain KOPRI 25157*	96.09%
Root	KC554446.1.1516	0.01%	6	Connectors	*Actinocorallia longicatena strain IMSNU 22180*	96.01%
Root	JF778694.1.1453	0.03%	4	Connectors	*Agromyces albus strain VKM Ac-1800*	98.89%

Root	KJ009457.1.1437	0.01%	4	Connectors	*Agromyces binzhouensis strain OAct353*	98.60%
Root	denovo3193	0.03%	4	Connectors	*Asticcacaulis solisilvae strain CGM1-3EN*	97.71%
Root	GQ407176.1.1450	0.02%	3	Connectors	*Bacillus sp. DV9-31*	100.00%
Root	JN178166.1.1557	1.14%	12	Connectors	*Bacillus lindianensis strain 12-4*	97.58%
Root	GQ280018.1.1459	0.50%	5	Connectors	*Bacillus sp. BJ-8*	100.00%
Root	FR746082.1.1413	0.18%	4	Connectors	*Bacillus sp. PG-2010-18*	100.00%
Root	HQ698291.1.1217	0.02%	6	Connectors	*Bradyrhizobium lupini strain USDA 3051*	96.80%
Root	DQ248243.1.1506	0.05%	37	Module_hubs	*Buttiauxella noackiae strain NSW 11*	92.58%
Root	JQ993930.1.1442	0.10%	34	Connectors	*Caballeronia cordobensis strain R-50210*	99.93%
Root	HM438001.1.1471	0.02%	23	Module_hubs	*Catenulispora subtropica strain TT 99-48*	87.92%
Root	HQ766669.1.1417	0.01%	4	Connectors	*Clostridium perfringens strain JCM 1290*	99.48%
Root	FR691488.1.1433	0.31%	31	Connectors	*Comamonas terrigena strain IMI 359870*	92.47%
Root	JF176742.1.1343	0.61%	20	Connectors	*Conyzicola lurida strain HWE2-01*	95.83%
Root	AB179523.1.1502	0.03%	17	Connectors	*Dokdonella fugitiva strain A3*	93.15%
Root	FJ950694.1.1472	0.02%	8	Connectors	*Escherichia-Shigella Escherichia fergusonii ATCC 35469 / Shigella sonnei strain CECT 4887 / Shigella flexneri strain ATCC 29903*	97.87%
Root	denovo2288	0.01%	28	Connectors	*Flavobacterium oncorhynchi strain 631-08*	95.14%
Root	FJ950564.1.1442	0.02%	20	Connectors	*Flavobacterium ginsengisoli strain DCY54*	97.45%
Root	FJ946569.1.1290	0.36%	28	Connectors	*Flavobacterium sp. BSw21750*	98.07%
Root	JF681476.1.1253	0.07%	13	Connectors	*Flavobacterium sp. TAPY6*	98.09%
Root	HE574373.1.1890	5.81%	19	Connectors	*Flavobacterium aquidurense strain WB 1.1-56*	95.92%
Root	KF611974.1.1426	0.03%	3	Connectors	*Flavobacterium qiangtangense strain F3*	96.08%
Root	JQ977390.1.1428	0.23%	13	Connectors	*Flavobacterium sp. Ama30*	100.00%
Root	FR774598.1.1302	0.04%	18	Connectors	*Flavobacterium sp. HME6659*	98.13%
Root	FM173271.1.1513	0.11%	9	Connectors	*Flavobacterium sp. BF.107*	100.00%
Root	FN668126.1.1484	0.07%	7	Connectors	*Flavobacterium buctense strain T7*	94.19%
Root	denovo33	0.05%	4	Connectors	*Flavobacterium sp. MK4S-17*	99.74%
Root	FR772074.1.1464	0.01%	33	Module_hubs	*Flavobacterium sp. R-38392*	100.00%
Root	HQ203850.1.1474	0.02%	33	Module_hubs	*Flavobacterium sp. strain AR-3-4*	97.99%
Root	denovo3533	0.04%	36	Module_hubs	*Flavobacterium sp. strain SYP-B2616*	98.97%

Root	KC355266.1.1438	0.03%	33	Module_hubs	*Flavobacterium denitrificans strain KUDC1759*	100.00%
Root	HM277555.1.1348	0.02%	31	Network_hubs	*Flavobacterium sp. HWG-A2*	96.22%
Root	JQ977221.1.1438	0.07%	14	Connectors	*Glaciihabitans tibetensis strain MP203*	97.92%
Root	GU201561.1.1452	0.24%	3	Connectors	*Herbaspirillum aquaticum strain IEH 4430*	97.70%
Root	FM209307.1.1493	0.01%	4	Connectors	*Herbaspirillum lusitanum strain P6-12*	95.78%
Root	HM266852.1.1342	0.01%	3	Connectors	*Ilumatobacter fluminis YM22-133*	91.62%
Root	GU591162.1.1448	0.01%	4	Connectors	*Lysinibacillus odysseyi 34hs-1 = NBRC 100172 strain 34hs1*	97.86%
Root	JF697399.1.1494	0.02%	8	Connectors	*Malikia granosa strain P1*	99.33%
Root	FQ658996.1.1356	0.03%	10	Connectors	*Marimicrobium arenosum strain CAU1038*	89.65%
Root	GQ379597.1.1200	0.04%	3	Connectors	*Massilia agri strain K-3-1*	97.52%
Root	HQ839786.1.1486	0.03%	9	Connectors	*Massilia lurida strain D5*	100.00%
Root	GU113051.1.1475	0.16%	4	Connectors	*Massilia namucuonensis strain 333-1-0411*	90.06%
Root	FM209302.1.1494	0.01%	7	Connectors	*Massilia namucuonensis strain 333-1-0411*	96.91%
Root	GQ264188.1.1442	0.06%	38	Connectors	*Mesorhizobium jarvisii strain ATCC*	99.36%
Root	JQ771197.1.1406	0.02%	8	Connectors	*Mesorhizobium acaciae strain RITF741*	99.48%
Root	FN421850.1.1371	0.03%	3	Connectors	*Methylobacterium marchantiae strain JT1*	98.09%
Root	FR683404.1.1497	0.01%	3	Connectors	*Methylotenera versatilis strain 301*	86.35%
Root	KP722591.1.1400	0.02%	9	Connectors	*Microbacterium rhizosphaerae strain CHO1*	100.00%
Root	HM337973.1.1338	0.25%	19	Connectors	*Mycobacterium hodleri strain DSM 44183*	97.83%
Root	HM332783.1.1356	0.01%	6	Connectors	*Nocardioides ginsengagri strain BX5-10*	96.03%
Root	GU208467.1.1450	0.01%	4	Connectors	*Novosphingobium arabidopsis strain CC-ALB-2*	97.55%
Root	AB505863.1.1513	0.01%	8	Connectors	*Paenibacillus sp. JJ-1b*	100.00%
Root	JN377674.1.1567	0.03%	35	Module_hubs	*Paenibacillus borealis strain B28*	100.00%
Root	GQ181054.1.1441	0.02%	31	Connectors	*Paraburkholderia phenazinium strain A 1*	97.43%
Root	JN832577.1.1471	0.07%	32	Connectors	*Paraburkholderia phymatum STM815*	97.08%
Root	GQ306173.1.1377	0.01%	7	Connectors	*Paraburkholderia solisilvae strain Y-47*	97.83%
Root	JF174728.1.1342	0.40%	9	Connectors	*Plantibacter flavus*	99.78%
Root	FJ946554.1.1290	1.27%	40	Module_hubs	*Polaromonas glacialis strain Cr4-12*	90.97%
Root	KC554646.1.1516	0.03%	7	Connectors	*Pseudarthrobacter oxydans strain DSM 20119*	96.38%
Root	FJ893917.1.1367	0.03%	4	Connectors	*Pseudomonas lactis strain DSM 29167*	96.05%

Root	denovo2018	0.02%	3	Connectors	*Pseudomonas lurida strain P 513/18*	96.72%
Root	denovo2664	0.01%	11	Connectors	*Pseudomonas libanensis strain CIP 105460*	96.47%
Root	AF159364.1.1496	0.05%	5	Connectors	*Rathayibacter caricis strain VKM Ac-1799*	100.00%
Root	AB456639.1.1331	0.04%	13	Connectors	*Rhizobium loessense strain CCBAU 7190B*	97.24%
Root	FR872466.1.1421	0.03%	13	Connectors	*Rhizobium aegyptiacum strain 1010*	98.10%
Root	FN421726.1.1361	0.09%	10	Connectors	*Rhizobium soli DS-42*	99.85%
Root	HQ856409.1.1448	0.03%	18	Connectors	*Rhizobium rosettiformans W3*	96.40%
Root	JN697723.1.1382	0.10%	12	Connectors	*Rhizobium sp. R2-7*	100.00%
Root	FR753119.1.1437	0.10%	11	Connectors	*Rhizobium pisi strain DSM 30132*	97.42%
Root	HF678384.1.1265	0.18%	38	Module_hubs	*Rhizobium lusitanum strain P1-7*	99.83%
Root	JF124163.1.1302	0.03%	3	Connectors	*Rhizobium cauense strain CCBAU 101002*	97.24%
Root	FN421520.1.1400	0.12%	4	Connectors	*Rhodococcus cercidiphylli strain YIM 65003*	96.32%
Root	denovo1571	0.02%	6	Connectors	*Rhodococcus qingshengii strain djl-6-2*	95.97%
Root	FM161354.1.1520	0.99%	39	Connectors	*Rhodoferax ferrireducens T118*	98.48%
Root	KM187281.1.1404	0.16%	17	Connectors	*Schumannella luteola strain KHIA*	97.08%
Root	JF204571.1.1353	0.03%	3	Connectors	*Snodgrassella alvi wkB2*	86.92%
Root	KC554125.1.1538	0.03%	3	Connectors	*Solirubrobacter ginsenosidimutans strain BXN5-15*	92.03%
Root	HQ111163.1.1491	0.01%	3	Connectors	*Solitalea canadensis DSM 3403*	85.11%
Root	AY771797.1.1408	0.02%	4	Connectors	*Sphingomonas laterariae strain LNB2*	97.28%
Root	FM211709.1.1465	0.05%	22	Connectors	*Sphingomonas roseiflava strain MK341*	96.25%
Root	HM339544.1.1306	0.02%	10	Connectors	*Sphingomonas mali strain NBRC 15500*	97.70%
Root	GQ006310.1.1371	0.01%	3	Connectors	*Staphylococcus hominis strain ICC_10-1_SCI_contig_1*	96.44%
Root	FM212990.1.1502	1.16%	43	Connectors	*Thiobacillus thiophilus strain D24TN*	93.03%
Root	HQ178879.1.1433	0.04%	7	Connectors	*Variovorax boronicumulans NBRC 103145 strain BAM-48*	97.61%

TABLE SⅥ

The composition of endophytic key microbiota in wheat leaves and roots filtered from the leaf and root co-occurrence networks.

Plant part	Genus	Relative abundance	OTU number	degree
Leaf	*Flavobacterium*	0.12%	2	24
Leaf	*Chryseobacterium*	0.05%	2	23
Leaf	*Pseudomonas*	0.01%	1	14
Leaf	*Tardiphaga*	0.03%	1	5
Leaf	*Acidovorax*	0.88%	1	4
Leaf	*Bacillus*	0.02%	1	3
Root	*Flavobacterium*	6.92%	16	328
Root	*Rhizobium*	0.60%	8	118
Root	*Paraburkholderia*	0.10%	3	70
Root	*Mesorhizobium*	0.08%	2	46
Root	*Paenibacillus*	0.04%	2	43
Root	*Thiobacillus*	1.16%	1	43
Root	*Polaromonas*	1.27%	1	40
Root	*Rhodoferax*	0.99%	1	39
Root	*Buttiauxella*	0.05%	1	37
Root	*Sphingomonas*	0.09%	3	36
Root	*Caballeronia*	0.10%	1	34
Root	*Comamonas*	0.31%	1	31
Root	*Acidovorax*	0.01%	1	27
Root	*Bacillus*	1.84%	4	24
Root	*Catenulispora*	0.02%	1	23
Root	*Massilia*	0.24%	4	23
Root	*Conyzicola*	0.61%	1	20
Root	*Mycobacterium*	0.25%	1	19
Root	*Pseudomonas*	0.06%	3	18
Root	*Dokdonella*	0.03%	1	17
Root	*Schumannella*	0.16%	1	17
Root	*Glaciihabitans*	0.07%	1	14
Root	*Marimicrobium*	0.03%	1	10
Root	*Rhodococcus*	0.14%	2	10
Root	*Microbacterium*	0.02%	1	9
Root	*Plantibacter*	0.40%	1	9
Root	*Agromyces*	0.04%	2	8
Root	*Escherichia-Shigella*	0.02%	1	8
Root	*Malikia*	0.02%	1	8
Root	*Herbaspirillum*	0.25%	2	7
Root	*Pseudarthrobacter*	0.03%	1	7

Root	*Variovorax*	0.04%	1	7
Root	*Actinocorallia*	0.01%	1	6
Root	*Bradyrhizobium*	0.02%	1	6
Root	*Nocardioides*	0.01%	1	6
Root	*Actimicrobium*	0.19%	1	5
Root	*Rathayibacter*	0.05%	1	5
Root	*Asticcacaulis*	0.03%	1	4
Root	*Clostridium*	0.01%	1	4
Root	*Lysinibacillus*	0.01%	1	4
Root	*Novosphingobium*	0.01%	1	4
Root	*Ilumatobacter*	0.01%	1	3
Root	*Methylobacterium*	0.03%	1	3
Root	*Methylotenera*	0.01%	1	3
Root	*Snodgrassella*	0.03%	1	3
Root	*Solirubrobacter*	0.03%	1	3
Root	*Solitalea*	0.01%	1	3
Root	*Staphylococcus*	0.01%	1	3

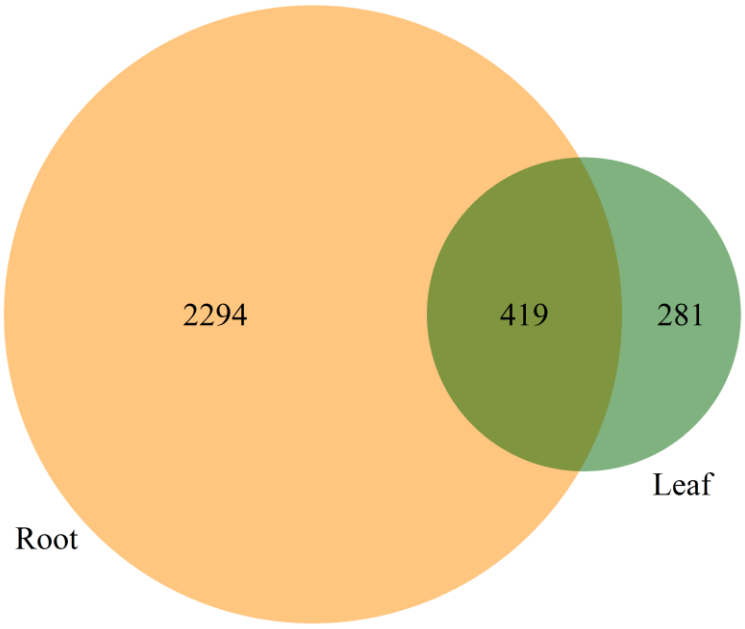

Fig. S1 Venn diagram showed OTUs overlap between wheat leaves and roots across all the samples.

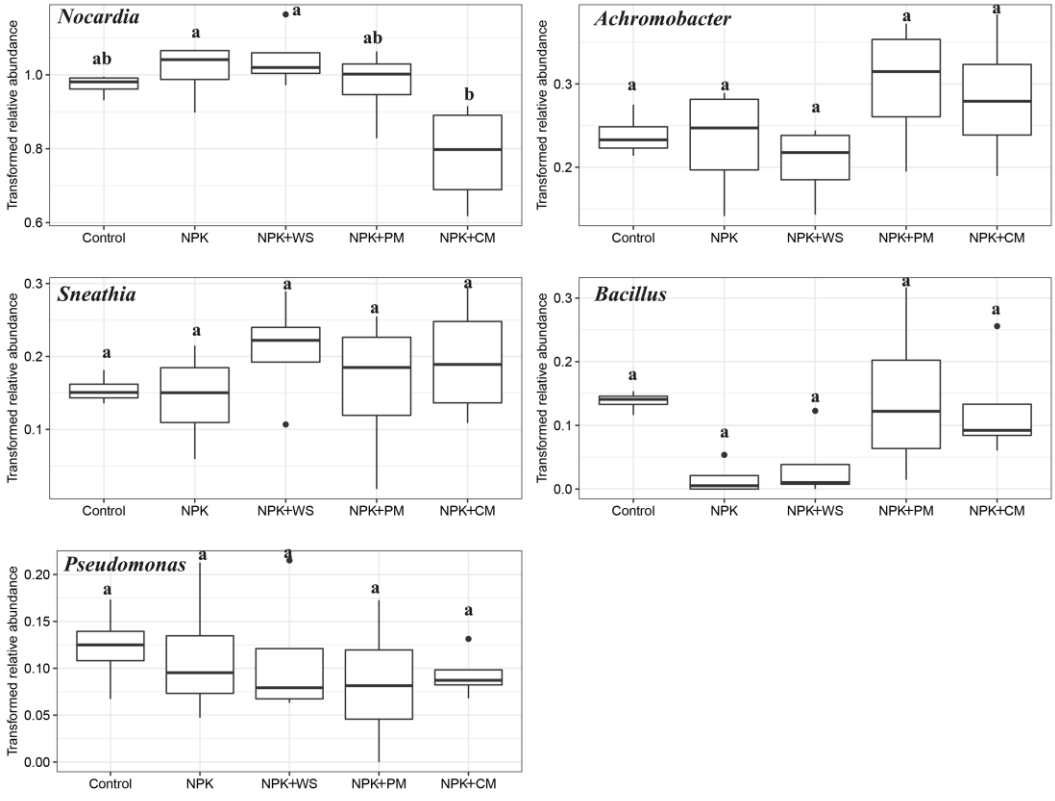

Fig. S2 Average relative abundance with an arcsine square root transformation of dominant endophytic bacterial genera across all treatments in winter wheat leaves. Different letters denote

significant difference between treatments detected by Turkey pairwise comparisons at $P < 0.05$. NPK: NPK fertilizer only; NPK+WS: NPK fertilizer plus wheat straw; NPK+PM: NPK fertilizer plus pig manure; NPK+CM: NPK fertilizer plus cow manure.

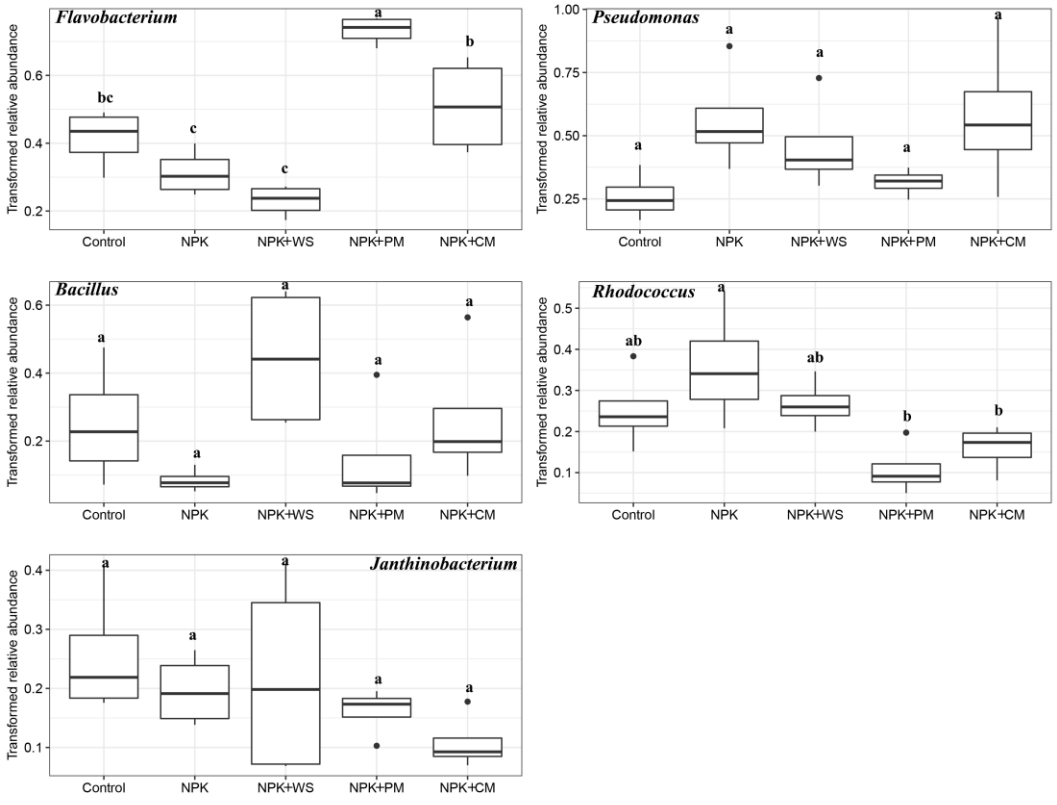

Fig. S3 Average relative abundance with an arcsine square root transformation of dominant endophytic bacterial genera across all treatments in winter wheat roots. Different letters denote significant difference between treatments detected by Turkey pairwise comparisons at $P < 0.05$. For abbreviations, see Fig. S2.

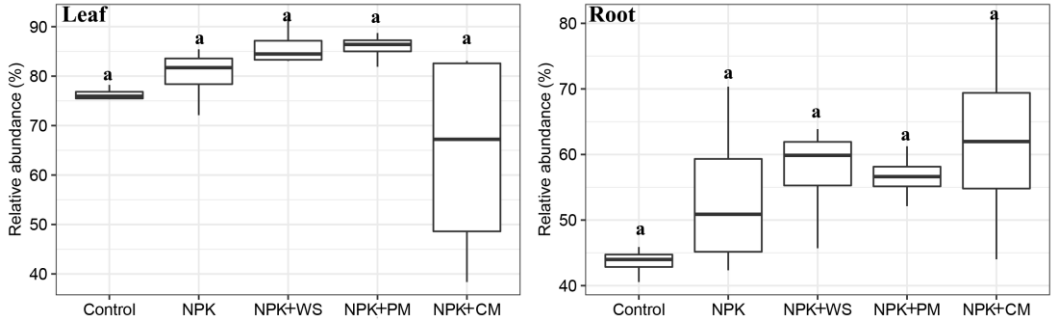

Fig. S4 The total relative abundance of abundant microbiota collected by all the OTUs with relative abundance more than 1.0% across all treatments in winter wheat leaves and roots, respectively. Different letters denote significant difference between treatments detected by Turkey

pairwise comparisons at $P < 0.05$. For abbreviations, see Fig. S2.

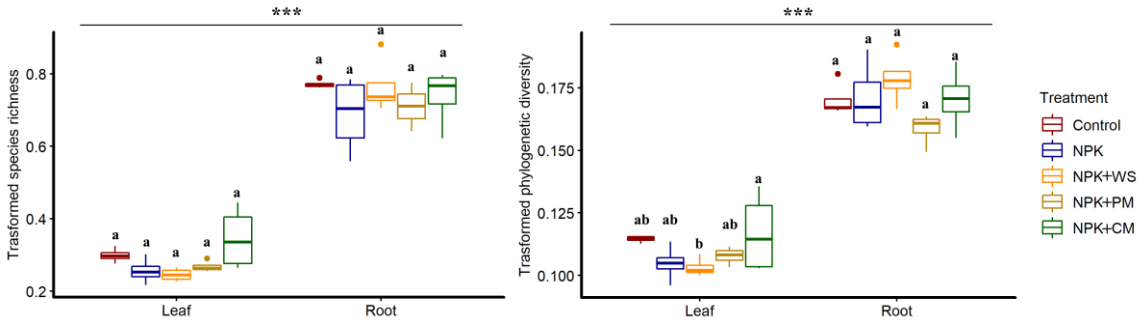

Fig. S5 Alpha diversity indices (species richness and phylogenetic diversity at the 4,700 seqs/sample rarefaction depths) of endophytic bacterial communities in wheat leaves and roots under fertilizations. Significant effects are indicated with asterisks (***P<0.001). Similarly, for each plant part we conducted separate ANOVAs testing the effects of treatments. Different letters denote significant difference between treatments detected by Turkey pairwise comparisons at $P <$ 0.05. For abbreviations, see Fig. S2.