Keystone microbiome in the rhizosphere soil reveals the effect of long-term conservation tillage on crop growth in the Chinese Loess Plateau

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Abstract

Purpose Keystone taxa play an important role in soil nutrient cycling and crop growth and can be influenced by soil tillage. We investigated the composition of keystone taxa and their relationships with soil properties under different long-term tillage practices.

Methods Four tillage treatments (i.e., CT, conventional tillage; NT, no tillage with mulch; RT, reduced tillage; and SS, subsoiling with mulch) were maintained for twenty-one years. Co-occurrence network (CoNet) was constructed to identify the keystone taxa, and redundancy analysis (RDA) was carried out to explore the relationships between keystone taxa and soil properties under the four tillage practices at two growth stages (elongation and grain filling stages) of winter wheat.

Results Compared with CT, RT had no significant effect on the microbial community and the keystone microbiome, while NT and SS remarkably altered the microbial community structure and the keystone microbiome at both growth stages. Massilia was the keystone genus under CT and RT, while Sphingomonas, Asanoa and Hoeflea were the keystone genera under NT and SS. RDA results showed that keystone genera were significantly correlated with soil organic carbon (SOC), dissolved organic carbon (DOC) and microbial biomass nitrogen (MBN) at both stages, especially at the elongation stage. Our results further revealed that the effects of NT and SS on crop growth were significant.
growth might be related to the changes in keystone microbiome.  

**Conclusion**  Our study suggests that NT and SS may contribute to the development of sustainable agricultural production in the Chinese Loess Plateau.

**Keywords** Long-term conservation tillage · Co-occurrence network · Redundancy analysis · Keystone microbiome · Winter wheat

**Introduction**

Winter wheat (*Triticum aestivum* L.) is one of the largest and most important food crops in China (Sun et al. 2018a), and its yield contributes more than 70% of the agricultural production in the semi-arid area of China (Xia et al. 2020). The Loess Plateau, spanning 64 million hectares in northwestern China, is a major farming area (Chen et al. 2015). Low nutrient utilization efficiency and high nutrient losses (especially N and P) constrain winter wheat yield improvements and complicate the development towards sustainable agriculture in this region (Niu et al. 2020). Therefore, improving nutrient use efficiency is essential to increase winter wheat production (Ali et al. 2019; Jin et al. 2008; Yan et al. 2020).

In many semi-arid regions, conversion from conventional tillage to conservation tillage is a promising strategy to improve soil structure (Martínez et al. 2013; Pareja-Sánchez et al. 2017), preserve soil water (Jin et al. 2007, 2008), and enhance the accumulation of SOC (Sun et al. 2018a; Thomas et al. 2019; Wang and Zou 2020). For instance, it has been shown that long-term no tillage with mulch (NT) improved the capacity of the soil to store and conserve water, to increase the content of dissolved organic carbon and available nutrients through retaining more crop residues (Singh et al. 2019). Subsoiling with mulch (SS) also proved to be an effective strategy to increase soil water content by stimulating water infiltration, and increasing soil carbon and nitrogen concentrations (Wang et al. 2020b). These increases in water storage capacity, organic carbon and nutrients are the most important factors contributing to winter wheat production enhancement (Lu and Liao 2017; Sun et al. 2018a; Yang et al. 2018).

Rhizo-bacterial activities largely determine crop development, nutrient absorption, and soil health (Mickan et al. 2019). Crop growth promoting effects of rhizo-bacteria have been proven in e.g. tomato (Ngoma et al. 2013), rice (Rangijaroen et al. 2015), maize (Naveed et al. 2014), and wheat (Duran et al. 2014). In addition, rhizo-bacteria create an interface between soil and crop roots (Semenov et al. 2020). In most cases, rhizo-bacteria are involved in the decomposition processes of soil organic matter and release of nutrients, thus promoting crop growth (Wang et al. 2017a). In turn, plants select unique rhizosphere microbial species, varying over the growing season following variations in root exudates, which ultimately improve available of soil nutrients (Guo et al. 2020). Therefore, management of rhizo-bacteria offers an opportunity to enhance the sustainability of the agroecosystem. However, optimization of a specific and useful rhizo-bacterium is a daunting task given the complexity of the interactions among microorganisms (Deng et al. 2012). Agler et al. (2016) developed the concept of “keystone taxa” to describe the taxa that were closely associated with many other taxa within the soil microbiome. Their findings prove that such highly connected microbes are important for plant health, as they mediate the feedback of plant and microbiome. In terms of this view, keystone taxa might recruit beneficial microorganisms or prevent invasion of pathogens in order to improve their fitness, for the benefit of the whole agricultural system. Focusing on keystone taxa is expected to more efficiently organize information about rhizo-bacterial interactions, to simplify the analysis of the microbiome, and to identify the key role of individual species within the microbiome (Herren and McMahon 2018). To date, keystone taxa have been identified in many environments such as forests (Hartmann et al. 2014), deserts (Zhou et al. 2020), and grasslands (Banerjee et al. 2018a), by defining the degree of node-specific interactions between taxa within co-occurrence networks (Fisher and Mehta 2014). However, studies on keystone taxa in semi-arid farming regions such as the Chinese Loess Plateau are lacking. Besides, although soil microbiome play an important role to regulate soil nutrient use efficiency (Toju et al. 2018), the underlying mechanisms how soil microorganisms, especially the keystone taxa, affect the soil nutrient use efficiency under long-term conservation tillage practices is still unclear.

To the best of our knowledge, previous studies on agroecosystems have mainly focused on the effect of
tillage practices on the rhizo-bacterial community composition (Tyler 2019; Xia et al. 2020; Wang et al. 2016). These studies consistently indicated that the relative abundances of Proteobacteria and Actinobacteria were increased by conservation tillage (e.g., NT, SS and RT), while Acidobacteria were reduced. Moreover, the shift in the rhizo-bacterial community depended on the number of years a tillage system was maintained. For example, studies that focused on the effects of five (Wang et al. 2016), six (Wang et al. 2017a), and ten (Wang et al. 2020c) years of NT on the rhizo-bacterial community structure indicated that the effects of NT on rhizo-bacterial composition changed between five and ten years (e.g., from Proteobacteria-dominated to Actinobacteria-dominated). Other research demonstrated that a fourteen-year RT harbored a higher relative abundance of Proteobacteria, due to the improvement of available water for this phylum (Tyler 2019). However, the longer-term effects (e.g., > twenty years) of different tillage practices on rhizo-bacteria remains unclear.

The growing season of a crop is another driver of change in the rhizo-bacterial community structure in agricultural systems (Spedding et al. 2004; Zhang et al. 2018). Root system development during the growing season and associated changes in rhizodeposition, i.e., exudation of crop roots of inorganic C into the soil, may alter the spatial distribution and quality of organic compounds, influencing the dynamics of the rhizo-bacterial community over time (Baetz and Martinoia 2014; Jones et al. 2009). Many researchers have investigated the interaction of tillage practices and crop growth stages in mediating the rhizo-bacterial community (Shi et al. 2013; Spedding et al. 2004), but the results of this research is often inconsistent. Shi et al. (2013) reported that the tillage effects on the rhizo-bacteria were dependent on the growing season, but Spedding et al. (2004) found there were no interactive effects between tillage and growing season. This thus calls for further research on the relationship between tillage and crop growth in regulating the rhizo-bacterial community.

We previously reported that eight consecutive years of NT and SS had pronounced positive effects on winter wheat (cv. Yumai 48) yield and soil physicochemical properties in the Loess Plateau of China (Jin et al. 2008). However, the underlying microbial mechanisms of these promising effects were not explored. In this study, we used high-throughput sequencing data to construct eight co-occurrence networks (CT-, NT-, RT-, and SS-CoNet at each of two growth stages, i.e., the elongation stage (Zadoks stage 31) and the grain filling stage (Zadoks stage 85) of winter wheat) to identify the keystone genera under different tillage practices maintained for twenty-one years. We hypothesized that 1) long-term tillage practices result in a unique keystone microbiome by altering soil physicochemical properties; 2) keystone microbiome developed under conservation tillage practices promote crop growth and increase yield. To test these hypotheses, we compared the relative abundance and composition of keystone genera and the complexity of microbial networks in different tillage treatments and then examined the correlations between soil available nutrients and keystone genera under long-term conservation tillage practices in order to elucidate the microbial mechanisms underlying the effect of long-term conservation tillage practices on the growth of winter wheat.

Material and methods

Site description

An experimental station was set up in Songzhuang Village, 25 km north of the city of Luoyang, Henan Province (113.08 East longitude, 34.58 North latitude), in the eastern part of the Loess Plateau, China, in 1999. In this region, Quaternary loess has accumulated to a thickness of approximately 50–100 m, and it has a loose and porous structure (Jin et al. 2009). The soil at the experimental site is a silty loam, classified as an Inceptisol according to Soil Taxonomy. In order to assess plot homogeneity, basic soil characteristics were analyzed based on their contribution to soil quality and crop performance; detailed information on basic soil characteristics is listed in Table S1.

Experimental design

Finding a homogeneous site that is large enough to accommodate true field replicates of different treatments is extremely challenging, and therefore compromises in experimental layout often have to be made. We delineated a very homogeneous area (no significant differences in soil properties prior to the start of the experiment) that was large enough to
layout five different treatments one next to each other, each treatment covering the same length of the area. This set-up was thus subject to pseudo-replication and space-for-time substitution limitations (Blois et al. 2013; Walker et al. 2010). Because of the homogeneity of the plot, statistical analysis of the effects of different soil management practices can be done using replicated samples from single plots, which reduces the total size of the experimental field and hence variability (Zhang et al. 2006).

The experimental sites have five tillage practices, including conventional tillage (CT), no tillage with mulch (NT), reduced tillage (RT), subsoiling with mulch (SS) and two crops per year (TC). Each tillage practice was 3 m wide by 90 m long with a 0.5 m guard row on both sides. We only analyze the effect of CT, RT, NT and SS on soil characteristics in this study. All four tillage treatments were cropped with winter wheat (cv. Yumai 48) in a continuous cropping monoculture, and the different tillage practices were applied consistently on the same experimental sites from 1999 onwards. Detailed information about each tillage practice is given in Table S2. Equal fertilizer rates based on common fertilizer practices were applied in each treatment in each year: namely 150 kg N/ha (as urea) and 35 kg P/ha (as superphosphate). All fertilizer N was applied once as basal fertilizer before sowing. In CT, fertilizer was broadcast and then incorporated into the soil immediately before sowing. For NT, RT, and SS, fertilizer was applied at sowing by direct drilling. Protection against insects (mainly budworm) was ensured with the application of the systemic organophosphorus insecticide omethoate (C₉H₁₂NO₄PS) at a dose of 1125 mL active compound ha⁻¹.

Sampling strategy

Rhizosphere soil samples and winter wheat plants were collected from each tillage practice at each growth stage. Briefly, six plots (0.5 x 0.5 cm) were randomly selected in each tillage treatment. In each plot, twenty winter wheat plants were sampled and mixed to one sample. To reduce the differences in the effect of rooting depth and minimize disturbance of the plot, we collected the roots from the 0–20 cm depth only. Then, all roots of each plot were shaken vigorously to remove any soil not tightly adhering to them, and the rhizosphere soils were carefully separated from root surface using a sterile scalpel (Song et al. 2007; Wang et al. 2017b). Two soil cores (7.5 cm diameter) were collected and mixed to one rhizosphere soil sample from each plot, six rhizosphere soil samples were collected in each treatment. In total, 48 rhizosphere soil samples were collected (6 samples/tillage practice x 4 tillage practices x 2 growth stages). Rhizosphere soil samples were sieved through a 2-mm mesh, and were divided into three sub-samples, placed on dry ice, and rapidly shipped to the laboratory. One sub-sample was oven-dried and used to determine the soil moisture, a second sub-sample was air-dried to assess soil physicochemical properties, and a third sub-sample was immediately frozen at −80 °C for DNA extraction and 16S rRNA gene MiSeq-sequencing (Dong et al. 2017; Wang et al. 2020d; Zhou et al. 2020).

Analysis of winter wheat and rhizosphere soil sample properties

The twenty winter wheat plants per plot were oven-dried at 65 °C for 48 h and weighed to assess the dry biomass (root and above-ground biomass separately). Yield of winter wheat per hectare was calculated. Rhizosphere soil pH was measured using a pH meter (HI 2221 Calibration CheckPh/ORP Meter, Hanna Instruments, Woonsocket, RI, USA). Briefly, 5 mL distilled water was added to 1 g air-dried soil and placed in a shaker for 18 h before pH measurement. The soil water content (WT) was measured by drying 10 g soil at 105 °C for 12 h.

Dissolved organic carbon (DOC) was measured in the non-fumigated soil using a TOC/TN analyzer (Analytik Jena AG, Jena, Germany) by adding 0.5 M K₂SO₄ and shaking for 60 min. Microbial biomass carbon (MBC) was measured as the methods of Shokuhifar et al. (2021). Briefly, soil samples were fumigated with ethanol-free CHCl₃. Control samples (non-fumigated) were also assessed. Fumigated and non-fumigated samples were extracted with 0.5 M K₂SO₄. The extracts were bubbled with CO₂-free air to remove the CHCl₃ (Gregorich et al. 1990), and the difference in DOC between fumigated and non-fumigated soils was calculated using a conversion factor of 0.45 (Álvaro-Fuentes et al. 2013). Organic nitrogen (ON) was extracted with 20 mL chloroform and the microbial biomass nitrogen (MBN) was measured in 10 g of fumigated and non-fumigated soil; the soil
was extracted with 0.5 M K$_2$SO$_4$. The MBN content was calculated using a $k_N$ factor of 0.45 (Zhang et al. 2016). Soil organic carbon (SOC) was determined by dichromate oxidation of 0.5 g of air-dried soil in 20 mL concentrated H$_2$SO$_4$ and 5 mL K$_2$Cr$_2$O$_7$ (Cania et al. 2019). Total nitrogen (TN) was measured with an Element Auto-Analyzer (Vario MAX CN; Elementar, Langenselbold, Germany) (Zhang et al. 2016). Nitrate nitrogen (NO$_3^-$-N) and ammonium nitrogen (NH$_4^+$-N) were extracted following the method described by López-Bellido et al. (2014). Briefly, 50 mL of 2 M KCl was added to 10 g of dry soil, the soil suspension was filtered, and the concentrations of NO$_3^-$-N and NH$_4^+$-N in the extract were determined using a continuous flow colorimeter analyzer (QUAATRO; BranLuebbe, Norderstedt, Germany). The sum of NO$_3^-$-N and NH$_4^+$-N was defined as available nitrogen (AN). Soil total phosphorus (TP) and available phosphorus (AP) were determined using 0.5 M H$_2$SO$_4$-HClO and 0.5 M NaHCO$_3$, respectively, following the molybdenum blue method.

DNA extraction, PCR, and 16S rRNA gene MiSeq-sequencing

Environmental DNA was extracted using the E.Z.N.A. Soil DNA Kit (Omega Bio-tek, Inc., Norcross, GA, USA) according to the manufacturer’s protocols. The concentration and quality of DNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA). The 16S rDNA V3–V4 region was amplified with primers 338F and 805R according to the method of Essel et al. (2019) (Table S3). Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), following the manufacturer’s instructions. The MiSeq Reagent Kit v3 was used to construct Illumina libraries according to the manufacturer’s instructions. Sequencing was performed on an Illumina MiSeq PE300 instrument according to the standard protocols. After sequencing, forward and reverse Illumina reads were paired using PEAR (v0.9.6) (Zhang et al. 2014). Barcode and primer sequences were removed using cutadapt (v1.91) (Martin 2011). Sequences shorter than 200 bp were removed after quality control. FLASH software was used to merge pairs of reads. The sequences were further analyzed using USEARCH v5.2.32 to filter and denoise the data by clustering sequences with <3% dissimilarity, and were submitted to the NCBI Sequence Read Archive (SRA) (http://www.ncbi.nlm.nih.gov/bioproject/770471) with accession number PRJNA770471. Operational taxonomic units (OTUs) were clustered at a ≥97% similarity threshold using the UPARSE pipeline (Schlatter et al. 2019).

Construction of co-occurrence networks

Eight co-occurrence networks (CoNet) for six replicates were separately constructed (Dong et al. 2017; Li et al. 2021; Sengupta and Dick 2015) based on the relative abundance of OTUs using CoNet software (version 1.1.1 beta) (Faust and Raes 2016) to assess the effect of different tillage practices on the microbial network complexity and keystone genera at two growth stages of winter wheat. Cytoscape v3.7.2 was used to visualize the network. Pearson, Spearman, mutual information, Bray–Curtis, and Kullback–Leibler metrics were used to identify the co-occurrence relationships among keystone genera. NetworkAnalyser tool was performed to obtain the topological characteristics, including the total number of nodes, total number of links, number of positive links, Betweenness centrality, Closeness centrality, Clustering coefficient, Neighborhood connectivity and Network centralization. In the network, nodes were taxa representing OTUs grouped at a specific level (e.g., OTU level in the present study). The edges that connect these nodes represent correlations between OTUs. Degree represents the number of direct correlations to a node in the network (Agler et al. 2016; Ma et al. 2016; Toju et al. 2018). In this analysis, nodes with a high degree (> 4), indicating that an OTU was directly connected to more than 4 other OTUs, were defined as “keystone genera”.

Statistical and bio-informatics analysis

Data reported for soil properties, winter wheat biomass and yield are mean values from six replicates. The effect of tillage was tested using ANOVA. Tukey’s test was used when the effects of the tillage treatments were significant. Differences at $P < 0.05$ level were considered significant. All statistical analyses were performed by IBM’s SPSS (version 20; SPSS Inc., Somers, USA).
Shannon, Chao1, observed_species, PD_whole_tree and Good’s coverage indexes were calculated by Mothur (v 1.39.5). Each OTU was classified using the RDP classifier based on the SILVA database after rarefaction. Non-metric multidimensional scaling (NMDS) was performed on the Bray–Curtis dissimilarity distance matrix, and significance of clustering was determined with permutational multivariate analysis of variance (PERMANOVA, 999 permutations) using the vegan package in R 3.6.1 (Higgins et al. 2020). Redundancy analysis (RDA) based on Spearman’s correlation coefficient was performed in R 3.6.1 using vegan to explore the relationships between keystone genera and environmental variables under different tillage practices at two growth stages.

Results

Soil physicochemical properties and winter wheat biomass and yield

There were no significant differences in soil pH among different tillage practices at both growing periods (Table 1). WT, SOC, TN, TP, DOC, AN, MBC and MBN were similar between RT and CT, but significantly higher under NT and SS compared with CT (P < 0.05). At the elongation stage, the contents of DOC, MBC and MBN were significantly higher by 153.6%, 42.1% and 93.9%, respectively, under NT, and by 192.2%, 54.6% and 106.7%, respectively, under SS, compared with those under CT. At grain filling stage, the contents of DOC, MBC and MBN were significantly higher by 142.5%, 32.6% and 57.6%, respectively, under NT, and by 149.1%, 29.6% and 63.5%, respectively, under SS.

There was no significant difference in winter wheat biomass between CT and RT, whereas a significant increase was observed under NT and SS at both stages (P < 0.05) (Fig. S1a). The biomass at the elongation stage was 28.1% higher for NT and 31.4% higher for SS treatment than for CT. At grain filling stage, the biomass was 8.5% was higher for NT and 10.6% higher for SS than for CT. Yield of winter wheat was also significantly higher for NT and SS (P < 0.05), by 5.3% and 6.1%, respectively, compared with CT (Fig. S1b).

Table 1 Soil properties under different tillage practices and growth stages

| Soil properties | Elongation stage | Grain filling stage |
|-----------------|------------------|---------------------|
|                 | CT               | RT                  | NT                  | SS                  | CT               | RT                  | NT                  | SS                  |
| pH              | 7.89 ± 0.22aA    | 8.19 ± 0.08aA       | 8.31 ± 0.06aA       | 8.21 ± 0.06aA       | 8.06 ± 0.07aA      | 8.25 ± 0.04aA     | 8.32 ± 0.02aA       | 8.28 ± 0.03aA       |
| WT (%)          | 5.71 ± 0.13bA    | 6.27 ± 0.07bA       | 7.28 ± 0.08aA       | 7.18 ± 0.04aA       | 5.48 ± 0.32bA      | 6.56 ± 0.25bA     | 7.46 ± 0.08aA       | 7.47 ± 0.33aA       |
| SOC (g/kg)      | 7.55 ± 0.11bA    | 6.75 ± 0.11bA       | 9.43 ± 0.10aA       | 9.57 ± 0.29aA       | 6.58 ± 0.27bA      | 5.37 ± 0.32bA     | 8.59 ± 0.39aA       | 9.44 ± 0.39aA       |
| TN (g/kg)       | 0.52 ± 0.02bA    | 0.53 ± 0.02bA       | 0.88 ± 0.05aA       | 0.97 ± 0.02aA       | 0.53 ± 0.03bA      | 0.54 ± 0.02bA     | 0.96 ± 0.03aA       | 0.97 ± 0.04aA       |
| TP (g/kg)       | 0.92 ± 0.01bA    | 0.83 ± 0.031bA      | 1.24 ± 0.07aA       | 1.12 ± 0.06aA       | 1.00 ± 0.04bA      | 0.91 ± 0.01bA     | 1.04 ± 0.04aA       | 1.10 ± 0.03aA       |
| DOC (mg/kg)     | 8.67 ± 0.32bB    | 9.38 ± 0.18bB       | 22.0 ± 0.38aB       | 25.3 ± 0.29aB       | 12.3 ± 0.64aB      | 10.4 ± 1.26aB     | 29.7 ± 0.15aA       | 30.5 ± 0.27aA       |
| AN (mg/kg)      | 5.84 ± 0.09bB    | 4.99 ± 0.07bB       | 7.25 ± 0.05aB       | 7.59 ± 0.07aB       | 6.92 ± 0.22aB      | 6.65 ± 0.06bA     | 10.4 ± 0.07aA       | 10.6 ± 0.07aA       |
| AP (mg/kg)      | 32.8 ± 0.93bA    | 33.2 ± 0.17bA       | 20.2 ± 0.14aA       | 22.4 ± 0.39aA       | 25.5 ± 0.31bB      | 25.7 ± 0.26bB     | 19.8 ± 0.79aA       | 22.1 ± 0.48aA       |
| MBC (mg/kg)     | 58.5 ± 0.16bB    | 52.9 ± 0.99bB       | 83.1 ± 1.26bB       | 90.3 ± 0.22aB       | 244 ± 2.78aB       | 215 ± 1.02aB      | 324 ± 1.87aA       | 316 ± 1.95aA       |
| MBN (mg/kg)     | 8.41 ± 0.11bB    | 8.45 ± 0.25bB       | 16.3 ± 0.32aB       | 17.4 ± 0.17aB       | 16.8 ± 0.41bA      | 16.5 ± 0.23aB     | 26.5 ± 0.29aA       | 27.5 ± 0.22aA       |

CT, conventional tillage; RT, reduced tillage; NT, no tillage with mulch; SS, subsoiling with mulch. WT, water content; SOC, soil organic carbon; TN, total nitrogen; TP, total phosphorus; DOC, dissolved organic carbon; AN, available nitrogen; AP, available phosphorus; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen. Different letters in the same row (lowercase letters represent the tillage effect, and capital letters represent the growth stage effect) indicate significant differences among treatments (one-way ANOVA, P < 0.05); values represent mean ± standard deviation.
coverage values were higher than 0.94, indicating that the bacterial community was captured at this sequencing depth. The NMDS plots showed that the microbial samples under CT and RT were closely clustered, while NT and SS were closely clustered (stress = 0.042) (Fig. 1). Compared with CT, the community structures were different under NT and SS (Fig. 1). At phylum level, rhizo-bacteria were mainly composed of Proteobacteria, Actinobacteria and Acidobacteria (Fig. 2a). There was no difference in the relative abundance of Proteobacteria under all tillage practices at both stages. The relative abundance of Acidobacteria was significantly higher under NT and SS than under CT and RT, with values up to 0.22 and 0.20 for NT and SS, respectively, and 0.12 and 0.14 for CT and RT, respectively, at the elongation stage. The differences in relative abundance of this phylum among the different tillage methods was smaller at the grain filling stage, but its relative abundance was still significantly higher in NT and SS than in CT and RT ($P < 0.05$). The relative abundance of Actinobacteria showed a different result, reaching values of 0.20 and 0.21 for NT and SS, respectively, and values of 0.30 and 0.27 for CT and RT, respectively, at the elongation stage. At the grain filling stage, the relative abundances of this phylum were 0.30 and 0.31 for NT and SS, and 0.34 and 0.37 for CT and RT, respectively. At genus level, rhizo-bacteria were composed of Massilia, Streptomyces, Pseudarthrobacter and Nocardioides in all treatments at both stages (Fig. 2b). Their relative abundances were significantly decreased by NT and SS ($P < 0.05$). Massilia was the most dominant genus. At the elongation stage, its relative abundance was 0.07 and 0.06 in NT and SS, respectively, while it was higher for CT and RT, reaching values of 0.11 and 0.10, respectively. At grain filling stage, the relative abundance of Massilia was lower under NT (0.02) and SS (0.02) than that under CT (0.04) and RT (0.04). There were no significant differences in other genera among tillage practices in both stages.

Fig. 1 Non-metric multidimensional scaling analysis of the rhizo-bacterial community structure under tillage treatments across crop growth. CT, conventional tillage; NT, no tillage with mulch; RT, reduced tillage; SS, subsoiling with mulch; e, elongation stage; g, grain filling stage

Fig. 2 The relative abundance of phyla (a) and genera (b) under different tillage practices at the elongation and grain filling stages. CT, conventional tillage; RT, reduced tillage; NT, no tillage with mulch; SS, subsoiling with mulch
Keystone microbiome in the rhizosphere soil

Co-occurrence network (CoNet) analysis was used to explore the effect of tillage practices on the keystone microbiome and network complexity during both growth stages (Table S5). At the elongation stage, the number of nodes was 238 under CT, but was higher for the other tillage treatments: 240, 306 and 264 for RT, NT and SS, respectively. At the grain filling stage, the network of CT consisted of 308 nodes, while NT - and SS - networks consisted of 359 and 381 nodes, respectively. The network of RT consisted of 276 nodes. Links within a network represented interactions among different OTUs, with the fewest links being recorded in CT at the elongation stage (216) and the grain filling stage (345). The number of links was higher for NT (294) and SS (291), at the elongation stage, and increased to 403 (NT) and 408 (SS) at the grain filling stage, indicating that the network structure became more complex for NT and SS compared with CT.

According to the phylogenetic taxonomy of nodes in the network, two main phyla, including Proteobacteria and Actinobacteria, were detected in the four tillage treatments and two growth stages (Fig. 3; Table S6). At genus level, the composition of keystone taxa was similar for CT and RT, as well as for NT and SS, while in NT and SS they were distinctly different from those in CT and RT. *Iamia, Rubrobacter, Steroidobacter, Devosia, Massilia* and *Mesorhizobium* commonly existed in CT and RT networks, and *Sphingomonas* commonly existed in NT and SS networks at the elongation stage. At the grain filling stage, *Pseudarthrobacter* and *Saccharothrix* were found in both CT and RT networks, and *Asanoa* and *Hoeflea* were found in both NT and SS networks.

Correlations between keystone microbiome and environmental variables

Significant correlations were observed between some keystone genera and SOC, DOC and MBN under tillage practices during winter wheat growth \((P<0.01)\), which was revealed by redundancy analysis (RDA) (Fig. 4). At the elongation stage, the first axis and the second axis explained 43.84% and 2.47% of the variations in keystone microbiome. SOC, DOC and MBN positively correlated with *Sphingomonas, Steroidobacter* and *Solirubrobacter*, and negatively correlated with *Massilia, Devosia* and *Streptomyces* (Fig. 4a). At grain filling stage, the first axis and the second axis explained 29.24% and 1.53% of the variations in keystone microbiome. Significant positive correlations were recorded for *Sphingomonas, Steroidobacter* and *Solirubrobacter* with SOC, DOC and MBN, whereas these parameters negatively correlated with *Massilia, Devosia* and *Streptomyces*.

Discussion

Long-term conservation tillage shapes the rhizo-bacterial community structure

Previous studies have demonstrated that the rhizo-bacterial community structure differed in response to different tillage practices (Hartman et al. 2018; Sun et al. 2020; Wang et al. 2017c). In the present study, the observable differences between conservation tillage (NT and SS) and conventional tillage practice for rhizo-bacterial community structure could ascribe to the change in the relative abundances of Actinobacteria and Acidobacteria. The difference in Actinobacteria among tillage practices was attributed to the changes in relative abundance of dominant genera, including *Streptomyces, Pseudarthrobacter* and *Nocardioides*, which belonged to the phylum Actinobacteria. In Acidobacteria, all corresponding genera were unidentified taxa, which could be attributed to the lack of sequencing depth (Sun et al. 2018b). In general, there is an increase in the number of identified taxa with an increasing in sequencing depth (Zaheer et al. 2018). Although Good’s coverage indicated a suitable the sequencing depth, apparently sequencing depth would need to be further increased to cover all taxa. In addition, CoNet results showed
a reduction in the number of keystone genera with increasing community richness, revealing keystone genera are the drivers in shaping the community structure (Banerjee et al. 2018b; Herren and McMahon 2018). A reduction in the number of keystone genera weakened the competition between communities for nutrients, consequently benefitting the survival of some rare taxa (Berry and Widder 2014). For instance, *Massilia*, *Streptomyces*, *Pseudarthrobacter* and *Nocardioides* were identified as keystone genera under CT and RT, whereas these genera were absent in NT and SS, which led to remarkable changes in the complexity of the networks (Barberán et al. 2012). Consistently, the function of keystone genera displayed a similar pattern with network complexity in response to tillage practices. Species of *Sphingomonas* have been shown to be capable of promoting plant growth (Asaf et al. 2020; Bibi et al. 2012; Rangjaroen et al. 2015). Moreover, Chen et al. (2020) and Liu et al. (2021) indicated that *Sphingomonas* played a central role in promoting maize growth under straw mulching. In the current study, *Sphingomonas* was a keystone genus in both NT and SS, but not in CT or RT, indicating that NT and SS alter the role of this genus. Therefore, the transition in the rhizo-bacterial community after changes in tillage could be a result of changes in network complexity and function of keystone genera. Our future work will focus on plant growth promotion by *Sphingomonas* under NT and SS treatments.

The findings further indicated that the effects of NT and SS on the rhizo-bacterial community structure gradually decrease with winter wheat growth. The seasonal changes in community structure could be explained by the variations in the relative abundance of growth-sensitive phyla (Actinobacteria) and genera (*Massilia*), but also by the changes in the keystone genera and network complexity. This implied that growth dynamics can mediate the relationship between tillage and rhizo-bacterial community structure. Our results also indicated that a marginally effect of conservation tillage on the winter wheat biomass at the grain filling stage than at the elongation stage, which further suggests that the keystone genera relate to winter wheat growth stage. For instance, *Sphingomonas* was the keystone genus at the elongation stage rather than at the grain filling stage. This genus has been shown to solubilize P and hence increase plant available P, ultimately reducing soil available P (Rangjaroen et al. 2015). This might be due to the rate of plant P uptake being higher than the rate of P solubilization by *Sphingomonas* (Wahid et al. 2020). In the present study, we only analyzed the biomass of winter wheat roots. In future research, other root characteristics such as length and branching and their relations with rhizo-bacteria could be included.
to further elucidate how crop growth stage mediates the effect of tillage on the rhizo-bacterial community.

It is well documented that tillage effects on rhizo-bacterial community are highly associated with soil physical and chemical properties (Chang et al. 2021; Kaurin et al. 2018; Wang et al. 2017a, 2020a). The CT is known to lead to loss of soil nutrients because it largely disrupts the soil structure and removes crop residues (Bu et al. 2020), resulting in e.g. erosional losses. We also found that the contents of SOC and soil nutrient, including those of TN, TP, DOC, AN and AP, did not show significant differences between CT and RT. Under RT, one tillage operation was implemented instead of two tillage operations in CT, and thus this simple reduction in tillage intensity does not result in a measurable effect on soil properties. Our previous study conducted on the same site after eight years of tillage treatments showed a similar result (Jin et al. 2008). It is also noted that NT had an effect comparable to the effect of SS on these soil parameters. In NT, the absence of any tillage practices resulted in accumulation of nutrients, as was also the case in SS where soil is only loosened to break up compaction; thus a similar soil nutrient content was observed between NT and SS. NT and SS exhibited significantly higher SOC and nutrient contents (i.e., TN, TP, DOC and AN) compared with CT and RT, which might be attributed to the low soil disturbance and to the retention of crop residues on the soil surface (Wang et al. 2018). Rhizo-bacteria can be divided in different groups with respect to nutrient acquisition. For instance, a number of copiotrophic groups rapidly grow in soils with high nutrient concentrations; whereas oligotrophic groups rapidly grow in soils with low nutrient concentrations (Ofek et al. 2012). Thus, we suggest that soil nutrients are one of the factors mediating the changes in rhizo-bacterial community. The seasonal shift in rhizo-bacterial community under NT and SS related to the seasonal changes in soil nutrients, such as DOC.

Long-term conservation tillage drives keystone microbiome by altering SOC, DOC and MBN

In this study, the compositions of keystone genera were significantly different for NT and SS in comparison with CT and RT. This is expected and probably due to the differences in soil variables among tillage practices (Wang et al. 2020c, 2020d). Therefore, we further investigated the interactions between keystone genera and soil properties by redundancy analysis (RDA), showing that SOC, DOC and MBN were significantly correlated with a part of the keystone genera ($P < 0.01$). It is noteworthy that SOC, DOC and MBN were significantly higher under NT and SS than under CT and RT. These results together indicated that NT and SS select specific keystone genera by altering SOC, DOC and MBN. Several mechanisms could be responsible for this finding. Firstly, NT and SS minimize soil disturbance; in contrast, in CT and RT, inversion tillage is used (with different intensity). Lower soil disturbance under NT and SS increase aggregate stability and maintain residue cover, thus reducing surface runoff and soil loss, and leading to accumulation of soil organic matter (SOM) (Wang et al. 2020b). Secondly, soil temperature is the main driver of SOM mineralization (Razavi et al. 2016). Under NT and SS, the higher stubble length than under CT and RT, and the straw retention decreased soil surface temperature, slowed down the mineralization of SOM (Bowles et al. 2014). Increasing SOM directly increases the content of DOC and MBN (Evangelou et al. 2021). Although winter wheat straw was not mixed into the soil under NT and SS, the straw will decompose at the soil interface leading to a gradual supply of DOC and MBN in NT and SS treatments. In this study, SOC, DOC and MBN were positively correlated with Sphingomonas, and were negatively correlated with Massilia. This might be attributed to the fact that Sphingomonas is a copiotrophic bacterium, and high SOC, DOC and MBN promote the growth of this genus (Bell et al. 2018; Ma et al. 2018). In the case of Massilia, an oligotrophic bacterium, low SOC, DOC and MBN promote the growth of this genus (Swarnalakshmi et al. 2019). This may explain why Sphingomonas was a keystone genus under NT and SS, while Massilia was a keystone genus under CT and RT. However, other studies on winter wheat rhizosphere soil bacteria found that Thermomonospora is the keystone genus under NT (Lu et al. 2019). This may be due to differences in soil properties (e.g., SOC). Furthermore, Sun et al. (2020) reported that the keystone genus in maize rhizosphere soil in the Loess Plateau was Gaiella, implying that crop species shape keystone genera by altering soil properties. In conclusion, our results revealed that reducing the number of tillage operations by one under RT would not lead to significant changes in
soil properties, keystone genera, or even crop growth, whereas NT and SS selected beneficial taxa that for the crop growth by altering soil properties, and consequently enhanced crop growth. Although the role of keystone genera in promoting crop growth is well documented in other research, we only estimated the composition of keystone genera in this study, their mechanisms in promoting crop growth under conservation tillage therefore still need to be assessed.

This study also demonstrated that NT and SS effects vary across the growing season and that differences become smaller towards the grain filling stage, which was expressed by the differences in complexity of the network within key-stone phyla, indicating that the effects of NT and SS on keystone phyla are regulated by crop growth (Shi et al. 2013). This may be attributed to the variations in root exudates of a crop (Zhao et al. 2020). These authors found a negative correlation between the content of phenolics in root exudates and the relative abundance of Actinobacteria, with the phenolics content decreasing during the growing season. Therefore, we suggest that the increase in Actinobacteria at grain filling stage might be caused by a reduction in phenolics production. The root exudates can stimulate the accumulation of DOC and MBN (Cheng et al. 2014). According to these observations, we suggest that DOC and MBN have a weaker response to NT and SS at the grain filling stage. Consistently, our results showed smaller effects of NT and SS on DOC and MBN in this stage. Moreover, less significant correlations between keystone taxa and DOC and MBN were observed at the grain filling stage than at the elongation stage by the RDA analysis, which further explains the weak effects of NT and SS on the keystone taxa at the grain filling stage. Overall, NT and SS influenced keystone taxa through altering the content of SOC, DOC and MBN. Our future work should be focused on the root exudates under conservation tillage during winter wheat growth, to reveal how keystone taxa respond to crop growth under conservation tillage practices.

Conclusion

There was no significant difference in microbial community between RT and CT at both growth stages after twenty-one years at the present site. However, NT and SS significantly influenced the microbial community. In addition, the complexity of the microbial network was remarkably enhanced by NT and SS, meanwhile selecting more keystone genera that were beneficial for crop growth. NT and SS showed indirect effects on the composition of keystone genera by altering SOC, DOC and MBN. We suggest against sampling at a single stage throughout crop growth, as we found differences in the response of keystone genera to NT and SS between elongation stage and grain filling stage. Our study also revealed that NT and SS affect winter wheat growth by shaping unique keystone genera. Overall, NT and SS were suitable conservation regimes and may contribute to the development of sustainable agricultural production in the Chinese Loess Plateau. Our findings enhance the understanding of the role of long-term conservation tillage in altering winter wheat growth and knowledge of specific keystone genera in regulating the growth of winter wheat in the Chinese Loess Plateau.

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Authors’ contributions

Lijuan Jia conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft. Zhen Wang conceived and designed the experiments, performed the experiments, collected soil samples, analyzed the data, revised draft, and approved the final draft. Lei Ji and Tao Zhou provided experimental equipment, prepared figures and/or tables, and revised the draft. Stefaan De Neve contributed to the experimental design and interpretation of data. Paul C. Struik contributed to the interpretation of the data and the drafting of the paper. Yuqing Yao and Junjie Lv collected soil samples and provided experimental equipment. Ke Jin conceived and designed the experiments, performed the experiments, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

Data availability

Not applicable.

Code availability

Not applicable.

Declarations
Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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