LINKING SOIL BACTERIAL COMMUNITY AND CROP YIELD IN A WHEAT (*TRITICUM AESTIVUM* L.) - ALFALFA (*MEDICAGO SATIVA* L.) INTERCROPPING SYSTEM

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Abstract. Diverse intercropping has been utilized to improve crop productivity on agricultural fields. Beneficial plant rhizobacteria are associated with plant root surface and may increase yield. In the research, the bacterial communities in soils of monoculture and intercropping wheat and alfalfa (cv. Winter star) were studied using MiSeq sequencing of the 16S rDNA gene. Intercropping pattern improved wheat yield in the field. The dominant taxonomic groups in the rhizosphere soil were *Proteobacteria*, *Acidobacteria*, *Bacteroidetes*, *Actinobacteria*, *Planctomycetes*, *Chloroflexi* and *Nitrospirae* and these were present across 4 samples. Intercropping significantly affected the diversity and composition of bacterial communities compared to monoculture. The enrichment of bacterial communities such as the populations of *Rhizobiales*, *Burkholderiales*, *Pseudomonales* and *Bacillus* could be important factors contributing to yield increases in intercropping wheat. In addition, some populations, such as *Sphingomonadals* and *Xanthomonadales*, indicated contrary changes, their diversity declined in intercropping systems, meaning that these bacterial populations were affected by cropping patterns.
Keywords: *monoculture, intercropping, bacterial community composition, MiSeq, rhizosphere*

Introduction

Intercropping has been used for many years to grow two or more plants in the same area of land simultaneously (Vandermeer, 1992). Intercropping ecosystems have

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demonstrated to be better than monoculture in terms of yields as a consequence of intercropping can make better use of one or more agricultural resources in time and space, through different rooting depths or over a year, to maximum the access to nutrients (Ma et al., 2017; Ren et al., 2017; Sylvain et al., 2018). Furthermore, the advantage of intercropping systems in yield is due to the interaction between intercropped species on the above- and below-ground (Du et al., 2011; Hauggaard-Nielsen et al., 2001). There are more reports about interspecies above-ground than below-ground interactions for interspecies interactions (Vandermeer, 1992; Willey, 1999). However, the effects of below-ground may be greater than above-ground species interactions for intercrop productivity (Hanming et al., 2012; Yue et al., 2014). There are compact relationships between yield advantage and water content, root morphologies nutrient uptake and root-associated microbes in intercropped soils (Choudhary et al., 2016; Zhou et al., 2011).

Intercropping of leguminous crops and cereals is one of the most practical intercropping techniques (Hesler and Kieckhefer, 2018) for improving crop yields and land use efficiency (Bhatti et al., 2006). Therefore, perennial alfalfa and annual wheat were selected to set up intercropping system in our study. In consequence, the root morphological and physiological characteristics of alfalfa and wheat are very different, and co-cultivation of both species can improve the absorption of water and nutrients by the root system (Skelton and Barrett, 2005). Leguminous plants could improve harsh environmental conditions or the available resources for other adjacent species by transferring of symbiotically fixed nitrogen (N) (Jensen, 1996a) and dissolving of inorganic phosphorus (P) fixed in soil (Yan et al., 1996). Furthermore, intercropping may increase soil microbial diversity, which usually has a positive effect on crop productivity (Xin et al., 2016). Free-living microorganisms strongly regulate plant productivity by mineralizing and competing nutrients that maintain plant productivity (Van Der Heijden et al., 2008).

Microorganisms are ubiquitous in the environment and play an essential role in the global biogeochemical cycles that sustain all life on Earth (Su et al., 2012; Zarraonaindia et al., 2013). It is well known that soil microbes carry out fundamental processes that contribute to nutrient acquisition (Li et al., 2016, 2020), nitrogen cycling (Li et al., 2017), carbon cycling (Schimel and Schaeffer, 2012) and soil formation (Rillig and Mummey, 2006). Soil microbes are crucial regulators of plant productivity (Van Der Heijden et al., 2008), and plant community composition considerably influences the community composition of rhizosphere microbes. The aboveground trophic interactions have indirect effects on soil biota by affecting the quantity and quality of resources that plants produce (Wardle et al., 2004). The roots of different plant species are in direct contact in intercropping ecosystem, and the root-associated communities of both plants species can therefore interact. The resulting microbial community composition is likely to be a mixture of the species-specific communities but may be dominated by the community composition of one plant species (Song et al., 2007). However, while it is widely recognized that microbes perform crucial roles in biogeochemical cycling, the impact of soil microbes on plant productivity is still poorly understood. Therefore, in order to better understand the changes of bacterial communities of monoculture versus intercropped plants in soils, 16S rDNA gene-based MiSeq sequencing approach was employed in wheat/alfalfa intercropping system, which may contribute to the greater yield in intercropping compared with sole cropping.

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Materials and methods

Field plots

A field experiment was conducted at Heilongjiang Academy of Land Reclamation and Agricultural Sciences, Jiamusi city of Heilongjiang province, China (latitude, 46°46′N; longitude, 130°27′E) in 2014. The region has a typical temperate continental climate with an average annual temperature of -3.0~-1.5 °C and the mean temperature of 20 °C in July. The mean annual precipitation is 450~550 mm and nearly 59% of total rainfall is received by northwest monsoons from July-September. The active accumulated temperature (≥ 10 °C) is 2000~2800 °C per year, and a frost-free period of 115~130 days. The soil is classified as a meadow black soil. Soil samples contained organic of 3.9%, available nitrogen of 46.9 $mg \cdot kg^{-1}$, available phosphorus of 145.5 $mg \cdot kg^{-1}$, available potassium of 121.0 $mg \cdot kg^{-1}$, pH of 6.7.

The experimental design was a plot divided into three blocks (three replicates), each block being further divided into three plots. Each plot was used for one of the following cropping systems: (1) wheat monoculture, (2) alfalfa monoculture, and (3) wheat intercropped with alfalfa. The experiment covered an area of 405.8 m^2. Each plot unit comprised 12 rows that were 5 m long and 0.66 m wide, each 39.6 m^2 in size. Plots and blocks were separated from each other by 1-meter walkways. For the intercropped treatment, two alfalfa rows were intercropped with two rows of wheat. The single cropping plots consisted of 12 rows of one plant species. Edges of each plot were sown with a mix of wheat and alfalfa to minimize edge effects but these plants were not included in the harvest.

The wheat (*Triticum aestivum* L. cv. Kenfeng No.1) and alfalfa (*Medicago sativa* L. cv. Winter star) were sown manually on 10 and 14 June 2014, respectively. Seedlings in each row were thinned after emergence to leave a density of 30 plants m^{-2} for alfalfa and 600 plants for wheat. Prior to sowing, fertilizer in the form of $(NH_4)_2HPO_4$ (150 $kg \cdot hm^{-2}$) were applied and the soil was disked to a depth of 10 cm. A conventional herbicide treatment was applied.

The yield of wheat and alfalfa was investigated using the quadrat harvesting method, and was determined in August 2014. The plants were killed at 105 ℃ for 30 min and dried at 60 ℃ to a constant weight.

Soil samples were collected from three different sampling sites at the flowering stage on 26 July 2014. Non-rhizoshpere soil were removed by shaking the root gently, Rhizosphere soils, adhering to the roots (Nazih et al., 2001), were placed into sterile petri plates. Three random sampling points were chosen for each sampling plot. Nine random single samples of rhizosphere soil were collected and thoroughly mixed in order to obtain a composite sample. The soil samples were sieved (2 mm) and stored at -80 °C until DNA extraction.

DNA extraction and PCR amplification 16S rRNA

The genome DNA was isolated using an Omega Bio-Tek E.Z.N.A. Soil DNA Extraction Kit (Omega Bio-Tek, Atlanta, GA, USA) according to the manufacturer's instructions. The equality of extracted DNA was examined following electrophoresis in a 1% agarose gels. The V4-V5 regions of the 16S rRNA gene were PCR amplified by using barcoded fusion primers 515F (5'-GTGCCAGCMGCCGCGG-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT-3'). For each sample, three independent amplification reactions were performed. The reaction mixture (20 μL) contained 5 μM

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of each primer, ~10 ng of template DNA, 5× FastPfu PCR buffer, 2.5 mM dNTPs and 2.5 U of FastPfu DNA Polymerase (MBI, Fermentas, USA). The amplification conditions were: 95 °C for 3 min and 7 cycles of denaturation at 95 °C at 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s, followed by a final extension period at 72 °C for 10 min. During amplification, a negative control reaction (lacking template DNA) was included to check the experimental contamination. All reactions were performed in triplicate. The PCR products were detected by electrophoresis in a 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA).

MiSeq sequencing and data analysis

The Illumina MiSeq PE250 was applied to perform barcoded V4-V5 amplicons by Shanghai Majorbio Bio-Pharm Biotechnology (Shanghai, China). Raw fastq files were demultiplexed and quality-filtered using QIIME (version 1.17). Sequences were clustered and assigned to operational taxonomic units (OTUs) at a 97% similarity level using UPARSE (version 7.1). To assess phylogenetic affiliations, taxonomic ranks were assigned to each sequence using Ribosomal Database Project (RDP). Compositional differences between libraries were determined using distance matrices and LIBSHUFF comparisons (Singleton et al., 2001). Alpha diversity (Ace, Chao 1, Simpson and Shannon) was calculated with QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3). Venn diagrams of unique and OTUs (0.03 cut-off value) were drawn to highlight the similarities and shared sequences between the different analyzed samples. Principal Component Analysis (PCA) in genus level was performed using ggplot2 package in R software (Version 2.15.3). Hierarchical cluster (Heatmap) analyses were generated in MOTHUR using the gplots package of R software (Version 2.15.3).

Statistical analysis

We used SPSS for windows (version 19) to test for significance ($P < 0.05$) between treatments of relative abundances, Alpha diversity and richness of bacterial communities using Duncan post-hoc test at 95% confidence level. To determine the key factor(s) affecting microbial parameters, stepwise multiple regression analysis was applied using the probability criteria of $P < 0.05$ to accept and $P > 0.1$ to remove a variable from the analysis.

Results

Plant yields

The yield of wheat and alfalfa were measured in September 2014 (*Fig. 1*). The intercropping significantly increased wheat yield compared with monoculture ($P < 0.05$). The yield in the intercropped wheat was 39.65% higher than monoculture. However, intercropping systems slightly decreased the biomass of alfalfa and there was no significant difference between monoculture and intercropping treatments ($P > 0.05$).

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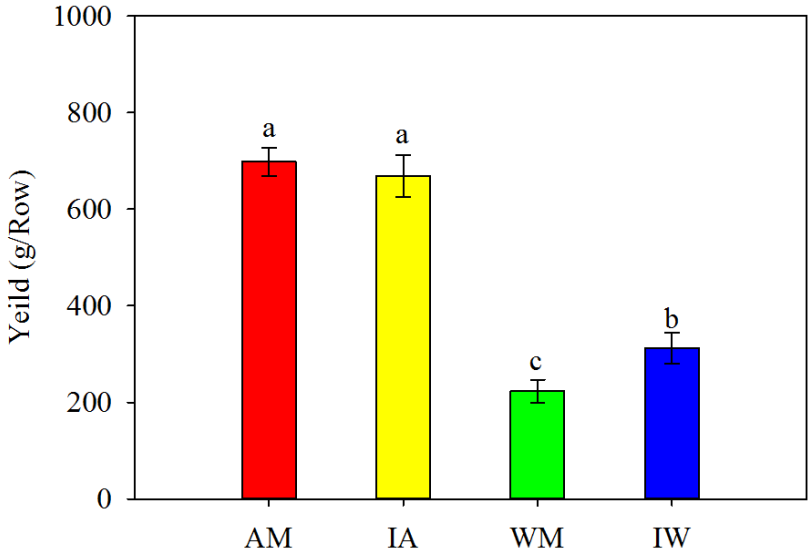

Figure 1. *The yield of wheat and alfalfa. Alfalfa monoculture (AM), intercropping alfalfa (IA), wheat monoculture (WM), and intercropping wheat (IW). Bars with different letters indicate significant difference at $P < 0.05$. The significant differences between the means were determined using Duncan post-hoc test*

Bacterial community analysis

A total of 148,259 paired-end ≥ 300-bp reads were acquired from all 12 samples, with 31,242, 42,231, 39,239 and 35,547 high quality reads at the monoculture alfalfa, alfalfa intercropping, monoculture wheat, and wheat intercropping soils, respectively (*Table A1* in the *Appendix*). The average read length was 396 bp. Based on 97% species similarity, 1,231, 1,188, 1,161 and 1,280 operational taxonomic units (OTUs) were obtained from monoculture alfalfa, alfalfa intercropping, monoculture wheat, and wheat intercropping soils, respectively.

Bacterial diversity and richness

To determine rarefaction curves and other measures of diversity, OTUs (operational taxonomic units) were identified at 3% genetic distance. Rarefaction curves indicated consistent differences in all 4 libraries (*Fig. A1* in the *Appendix*). At 3% genetic distances, almost all rarefaction curves reached saturation, indicating that the surveying effort covered almost the full extent of taxonomic diversity at this genetic distance.

The comparison of mean Chao 1 richness estimates of alfalfa rhizosphere soils and wheat rhizosphere soils showed no differences at genetic distances of 3% (421 OTUs and 424 OTUs, respectively) (*Table 1*). Analysis of differences of cropping pattern by at genetic distances of 3% showed that the intercropping patterns varied in the predicted number of OTUs ($P > 0.05$). The uniform conclusion was seen using the Ace richness index (*Table 1*). Moreover, the comparison of the mean Shannon diversity index of 4 libraries revealed that the highest bacterial diversity at analyzed genetic distances was found in intercropping wheat and alfalfa soils, followed by monoculture wheat and alfalfa. The predicted richness and diversity in the intercropping rhizosphere soils exceeded that of the corresponding monoculture soils. Meanwhile, an influence of plant species on bacterial diversity was observed. Wheat soils demonstrated higher diversity

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than corresponding alfalfa in different cropping patterns. Thus, both intercropping system and plant species impacted overall bacterial diversity and richness.

Table 1. *The diversity index of 4 libraries. Means of three replicates ± SE. Different letters following the mean values within each column indicates significant differences at P < 0.05*

Sample	0.97			
	Ace	Chao 1	Shannon	Simpson
Monoculture alfalfa	406.0 ± 12.83 a	403.3 ± 14.61 a	4.81 ± 0.029 a	0.0181 ± 0.0008 b
Alfalfa intercropping	420.3 ± 12.47 b	439.3 ± 25.84 b	5.13 ± 0.021b	0.0101 ± 0.0003 a
Monoculture wheat	401.7 ± 9.18 a	406.7 ± 14.27 a	5.11 ± 0.021 a	0.0104 ± 0.0003 a
Wheat intercropping	449.3 ± 10.34 b	441.7 ± 9.18 b	5.27 ± 0.025 b	0.0093 ± 0.0004 a

Distribution of taxa and phylotypes across 4 liberates

The 49,394 classifiable sequences were affiliated with 9 bacterial phyla (*Fig. A2*). The groups accounted for 97.31% of all sequences, and a few sequences (< 1%) could not be shown. The dominant phyla were as follows: *Proteobacteria*, *Acidobacteria*, *Bacteroidetes*, *Actinobacteria*, *Planctomycetes*, *Chloroflexi*, and *Nitrospirae*, representing 42.60, 25.10, 8.93, 4.92, 4.73, 4.15 and 3.72%, respectively, of all sequences that were classified below the domain level. These dominant bacterial phyla were shared in all samples (*Table 2*). Other sequences belonged to *Firmicutes*, *Gemmatimonadetes* and unclassified bacteria, and they were invariably found in very low proportions (< 2%). *Proteobacteria* accounting for 42.60% was the most dominant among the 9 phyla in all samples, regardless of the different samples. *Acidobacteria* was the second largest phylum in all groups accounting for 25.10%. The other 7 phyla sequences accounting for 8.93-1.07% (*Fig. A2*).

Comparative analysis of the 4 libraries revealed a distinct distribution of the bacterial phyla (*Table 2*). On average, *Proteobacteria* and *Actinobacteria* showed a higher relative abundance in alfalfa rhizosphere soils than in wheat rhizosphere soils, whereas *Bacteroidetes* and *Chloroflexi* showed the opposite pattern. The phyla of *Actinobacteria*, *Acidobacteria*, *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Bacteria_unclassified*, *Gemmatimonadetes* and *Nitrospirae* were found in variable proportions, depending on the use of monoculture or intercropping; Most of *Bacteroidetes*, *Bacteria_unclassified* and *Gemmatimonadetes* were found in alfalfa intercropping libraries ($P < 0.05$), whereas *Proteobacteria* and *Firmicutes* were present at higher percentages in monoculture alfalfa libraries ($P < 0.05$). Moreover, the relative abundances of *Actinobacteria* and *Firmicutes* in wheat intercropping were significantly higher than that of monoculture wheat ($P < 0.05$), whereas *Acidobacteria* and *Nitrospirae* were present at higher percentages in monoculture wheat libraries ($P < 0.05$).

Proteobacteria sequences

The dominant phyla across all 4 libraries were *Proteobacteria* representing 42.60%, which predominated in all 4 libraries and showed the greatest diversity. Four classes: α-*Proteobacteria*, β-*Proteobacteria*, δ-*Proteobacteria* and γ-*Proteobacteria*, were affiliated with the *Proteobacteria* phylum (*Fig. 2*). The β-*Proteobacteria* sequences were most abundant, representing 41.00% of the *Proteobacteria*. The α-*Proteobacteria*

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and γ-*Proteobacteria* were relatively abundant, representing 25.36 and 27.65% of the *Proteobacteria*, respectively. The γ-*Proteobacteria* sequences representing 5.99% of the *Proteobacteria*.

Table 2. *Relative abundance of the phylogenetic groups presents in the monoculture and intercropping soils. Note: a one factor (niche) ANOVA (Duncan post-hoc test) was applied on the relative distribution values and results are presented in the column entitled 'statistics'. ns: non-significant differences. The phylogenetic groups for which a significant or nearly significantly effect of the niche are presented. AM > IA means significantly more present in the AM soil than in IA soil. AM < IA means significantly more present in the IA soil than in AM soil. WM > IM means significantly more present in the WM soil than in IM soil. WM < IM means significantly more present in the IM soil than in WM soil*

	Alfalfa monoculture			**Intercropping alfalfa**				**Wheat monoculture**			**Intercropping wheat**			
	AM1	AM2	AM3	IA1	IA2	IA3	Statistics	WM1	WM2	WM3	IW1	IW2	IW3	Statistics
Proteobacteria	51.02	50.13	47.05	37.64	42.94	40.98	AM>IA (*P*=0.0106)	42.02	46.19	39.60	43.20	44.23	41.98	ns
Acidobacteria	18.93	22.38	23.69	21.99	25.66	26.99	ns	23.23	24.87	27.21	16.90	15.86	21.20	WM>IW (*P*=0.0237)
Actinobacteria	8.99	6.69	6.99	8.94	9.12	10.47	ns	5.52	4.18	5.06	10.02	8.79	7.17	IW>WM (*P*=0.015)
Bacteroidetes	2.89	3.56	3.78	8.13	5.79	5.83	IA>AM (*P*=0.0179)	10.57	7.46	8.76	6.37	7.29	7.04	ns
Chloroflexi	2.96	3.43	3.47	4.76	3.02	3.35	ns	3.42	3.62	5.41	6.14	7.11	5.23	ns
Firmicutes	5.63	6.29	6.95	2.53	1.86	1.76	AM>IA (*P*=0.0007)	1.78	1.58	1.47	5.02	4.14	3.89	IW>WM (*P*=0.0015)
Nitrospirae	2.72	3.82	2.40	4.13	2.83	2.86	ns	4.00	3.55	3.62	2.03	2.18	3.01	WM>IW (*P*=0.0171)
Planctomycetes	3.02	2.04	2.90	4.64	3.39	2.73	ns	5.12	4.46	4.61	4.78	3.61	3.91	ns
Bacteria_unclassified	0.23	0.27	0.32	3.45	2.67	2.46	IA>AM (*P*=0.001)	2.01	1.52	1.38	2.11	2.46	1.92	ns
Gemmatimonadetes	1.60	1.01	1.02	2.48	1.94	1.85	IA>AM (*P*=0.0336)	1.41	1.51	1.69	1.39	1.42	1.82	ns
Others	2.01	0.38	1.43	1.31	0.78	0.72	ns	0.92	1.06	1.19	2.04	2.91	2.83	IW>WM (*P*=0.006)

The α-*Proteobacteria* were relatively abundant and diversity. 5 orders were identified as being related to α-*Proteobacteria* (*Fig. 2A*). The *Rhizobiales* was the most abundant order in 4 libraries, representing 63.47%. The relatively abundant orders affiliated to α-*Proteobacteria* were *Caulobacterales*, *Rhodospirillales*, *Richettsiales* and *Sphingomonadals*, representing 9.82,11.42, 0.03 and 15.25%. Depending on the use of monoculture and intercropping, most of *Rhizobiales* and *Rhodospirillales* were found in intercropping alfalfa, whereas low abundance was found in intercropping wheat ($P < 0.05$). *Sphingomonadals* showed a contrary variation, presenting at higher abundances in intercropping wheat and lower abundances in intercropping alfalfa comparing with monoculture ($P < 0.05$), respectively. The comparison of relative abundances of *Caulobacterales* revealed no significant differences between intercropping and monoculture libraries ($P > 0.05$). 1 OTU was related to *Richettsiales* and identified in alfalfa monoculture libraries only.

The β-*Proteobacteria* were most abundant and diversity in 4 libraries. 8 orders were identified as being related to β-*Proteobacteria* (*Fig. 2B*). The *Burkholderiales* was the most abundant order in 4 libraries, representing 65.60%. The relatively abundant orders

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affiliated to β-*Proteobacteria* were *Nitrosomonadales*, SC-I-84 and *Methylophilales*, representing 13.91, 13.78 and 1.77%. Depending on the use of monoculture and intercropping, most of *Burkholderiales* and *Methylophilale* were found in monoculture libraries (WM and AM) ($P < 0.05$), whereas Beraproteobacteria_unclassified, *Nitrosomonadales* and SC-I-84 were present at higher abundances in intercropping alfalfa compared with monoculture alfalfa ($P < 0.05$). The comparison of relative abundances of TRA3-2 revealed no significant differences between intercropping and monoculture libraries ($P > 0.05$). 95 and 2 OTUs were related to *Hydrogenophilale* and B1-7BS, respectively, while *Hydrogenophilale* appeared in intercropping wheat libraries only and B1-7BS in alfalfa monoculture and wheat monoculture libraries.

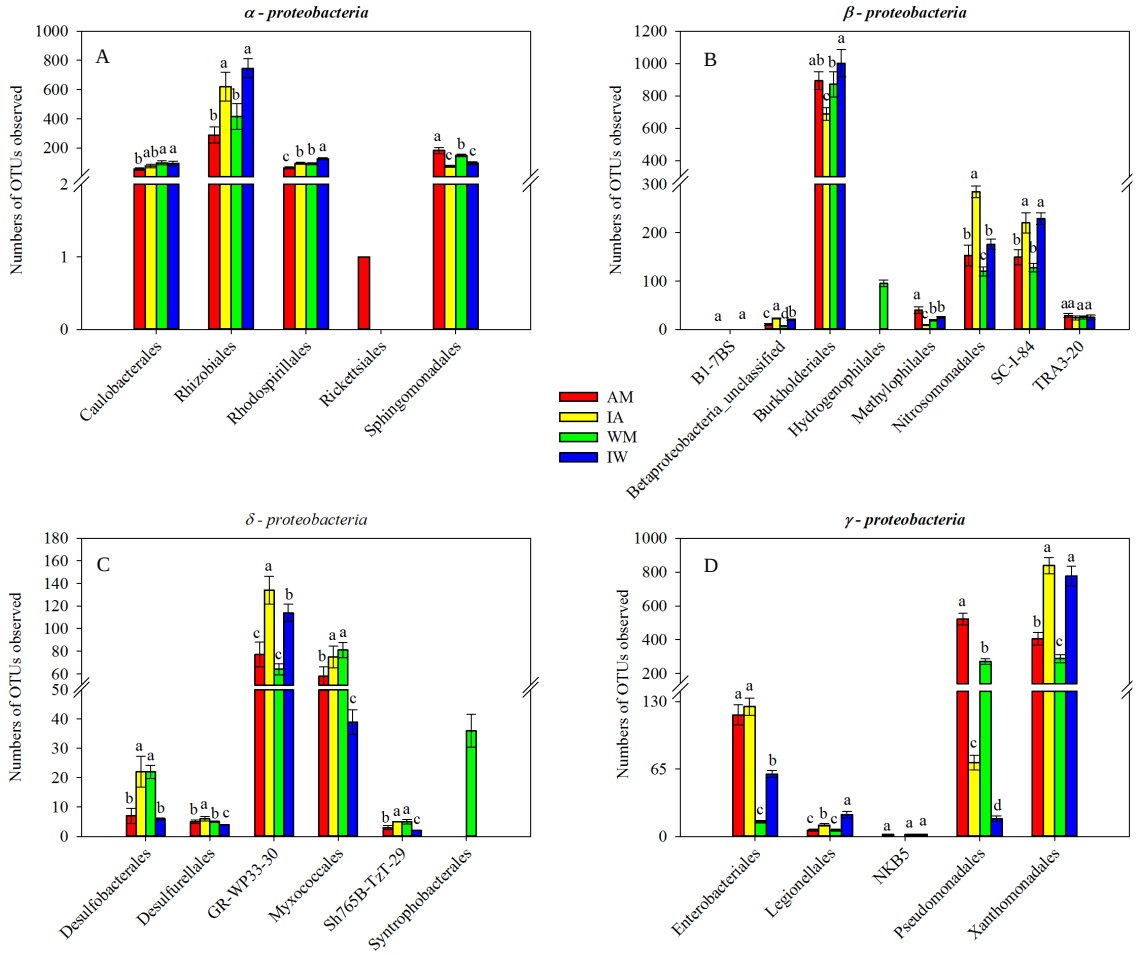

Figure 2. *Numbers of OTUs of Proteobacteria orders in 4 libraries. The significant differences between the means were determined using Duncan post-hoc test*

The δ-*Proteobacteria* were relatively abundant and diversity. 6 orders were identified as being related to δ-*Proteobacteria* (*Fig. 2C*). The GR-WP33-30 was the most abundant order in 4 libraries, representing 50.52%, with a relative abundance in intercropping alfalfa higher compared to corresponding monoculture alfalfa, whereas GR-WP33-30 was present at lower relatively abundances in intercropping wheat. The relatively abundant orders affiliated to δ-*Proteobacteria* were *Desulfobacterales* and *Myxococcales*, representing 7.40 and 32.86%. Depending on the use of monoculture and

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intercropping, most of *Desulfobacterales*, *Desulfurellales*, *Myxococcales* and *Sh765B-TzT-29* were found in intercropping alfalfa libraries ($P < 0.05$), whereas low abundance was found in intercropping wheat libraries ($P < 0.05$). 36 OTUs was related to *Syntrophobacterales* and identified in intercropping wheat libraries only.

The γ-*Proteobacteria* were relatively abundant and diversity. 5 orders were identified as being related to γ-*Proteobacteria* (*Fig. 2D*). The *Xanthomonadales* was the most abundant order in 5 libraries, representing 64.93%, with a relative abundance in intercropping alfalfa higher compared to corresponding monoculture alfalfa, whereas *Xanthomonadales* was present at lower relatively abundances in intercropping wheat. The relatively abundant orders affiliated to γ-*Proteobacteria* were *Pseudomonadales*, *Enterobacteriales*, *Legionllales* and NKB5 representing 24.77, 8.89, 1.24 and 0.17%. Depending on the use of monoculture and intercropping, most of *Pseudomonadales* were found in monoculture libraries ($P < 0.05$). The comparison of relative abundances of *Enterobacteriales* and NKB5 revealed no significant differences between intercropping and monoculture alfalfa ($P > 0.05$), whereas *Enterobacteriales* and *Legionllales* was present at higher abundances in intercropping wheat ($P < 0.05$). 6 OTUs was related to NKB5 and appeared in AM, IW, WM libraries, respectively.

Acidobacteria sequences

25.10% of the total clones were affiliated with the *Acidobacteria* phylum. These sequences were affiliated with 10 orders of *Acidobacteriales*, Subgroup 3, 4, 5, 6, 7, 10, 11, 17, 25 and no rank *Acidobacteria* (*Fig. 3*). The Subgroup 6, 4 and 3 were the most abundant order in 4 libraries, representing 66.01, 18.93 and 6.95%, respectively. The relative abundances of Subgroup 3 and 6 in intercropping alfalfa were higher than monoculture alfalfa ($P < 0.05$). A similar trend was also found by comparison of intercropping and monoculture wheat. In addition, sequences affiliated to Subgroup 5, 7, 17 and 25 were relatively abundant order in 4 libraries. The comparison of relative abundances of Subgroup 5 revealed no significant differences between intercropping and monoculture libraries ($P > 0.05$). We observed higher relative abundances of Subgroup 7 in intercropping libraries compared to corresponding monoculture libraries ($P < 0.05$). Depending on the use of monoculture and intercropping, most of Subgroup 17 was found in intercropping alfalfa libraries, and high abundance of Subgroup 25 was found in intercropping wheat libraries ($P < 0.05$). The abundance of Subgroup 10, 11, *Acidobacteriales* and no rank *Acidobacteria* were low, representing 0.80, 0.19, 0.30 and 0.42%.

Sequences of other relatively abundant groups

The *Bacteroidetes* were relatively abundant and diversity, accounting for 8.93% of the analyzed OTUs. These OTUs were affiliated with 4 orders of *Cytophagales*, *Flavobacteriales*, *Sphingobacteriales* and VadinHA17_norank (*Fig. 4*). The relatively abundances of *Cytophagales* in intercropping libraries were higher than monoculture, while *Flavobacteriales* and *Sphingobacteriales* in intercropping alfalfa were higher than monoculture. In addition, 80 OTUs was related to VadinHA17_norank and identified in IW libraries only.

The Actinobacteria had most abundant orders, affiliated with 11 orders of Acidimicrobiales, Corynebacteriales, Frankiales, Gaiellales, Micrococcales,

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Micromonosporales, Propionibacteriales, Pseudonocardiales, Solirubrobacteriales, Streptomycetales and Actinobacteria_norank (*Fig. 4*). The relatively abundances of Acidimicrobiales, Frankiales, Gaiellales, Propionibacteriale and Solirubrobacteriales in intercropping libraries were higher than monoculture.

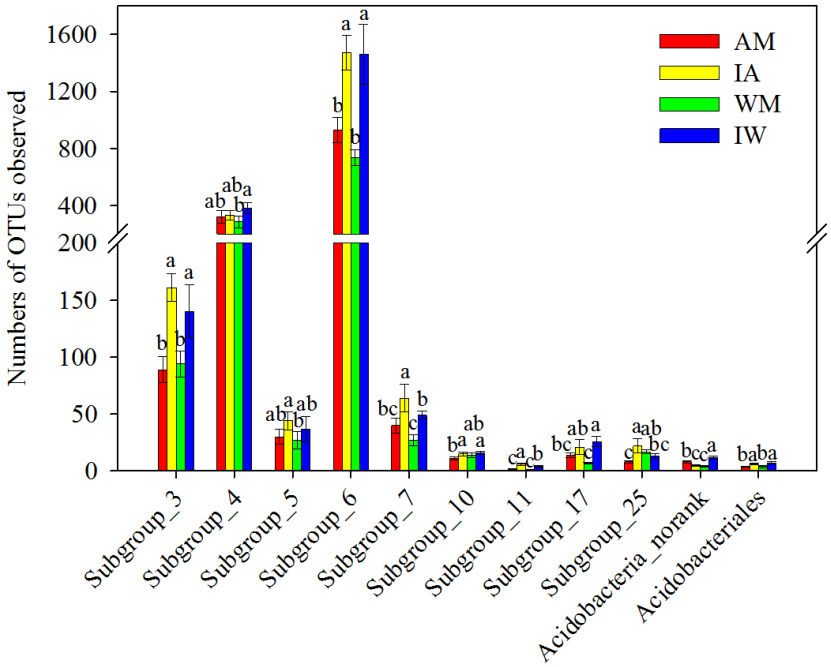

Figure 3. *Numbers of OTUs of Acidobacteria orders in 4 libraries. The significant differences between the means were determined using Duncan post-hoc test*

In addition, some orders affiliated to other phylum had significant differences between intercropping and monoculture libraries. Such as *Anaerolineales*, *Caldilineales*, *Clostridiales* (affiliated to *Chlorofiexi*) and *Lactobacillales* (affiliated to *Fimicutes*) showed a higher relative abundance in intercropping libraries compared with monoculture libraries, regardless of plant species (*Fig. 4*). Whereas, depending on the use of monoculture and intercropping, *Bacillus* (affiliated to *Fimicutes*) was present at higher relatively abundances in intercropping alfalfa only.

Shared bacterial OTUs

Venn diagrams revealed that the sum of total observed OTUs in the four soil samples was 475 (*Fig. 5*), and 307 OTUs were shared all of the soil samples. Moreover, the distribution of sequences demonstrated once again that each plant rhizosphere had its own microbial population.

PCA analysis

This was supported by the principal component analysis (PCA) with the weighted Unifrac distance (*Fig. 6*). Overall, the two PCA axes explained 81.41% of the variation between the different communities. The PCA score plot revealed that intercropping patterns significantly change bacterial communities in wheat and alfalfa rhizosphere soils. The intercropping (IA and IW) soil microbiota clustered separately from the

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microbiota of the monoculture (AM and WM) soils along principal components 1 (*Fig. 6*), suggesting that the application of intercropping pattern influenced the population structure of the soil bacteria.

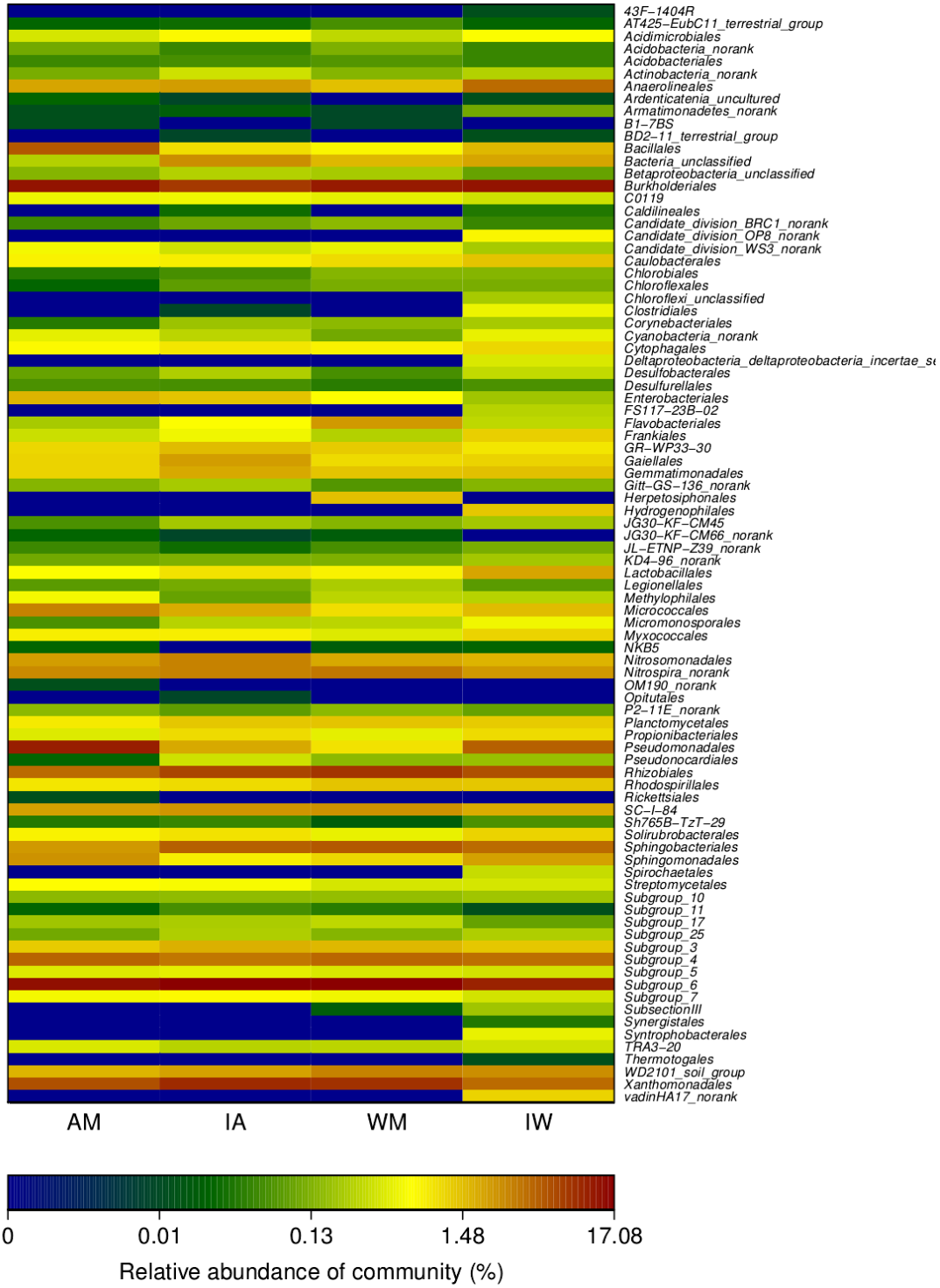

Figure 4. *Hierarchical cluster analysis of predominant orders among the 4 libraries. The color intensity of scale indicates relative abundance of each OTU read. Relative abundance was defined as the number of sequences affiliated with that OTU divided by the total number of sequences per sample*

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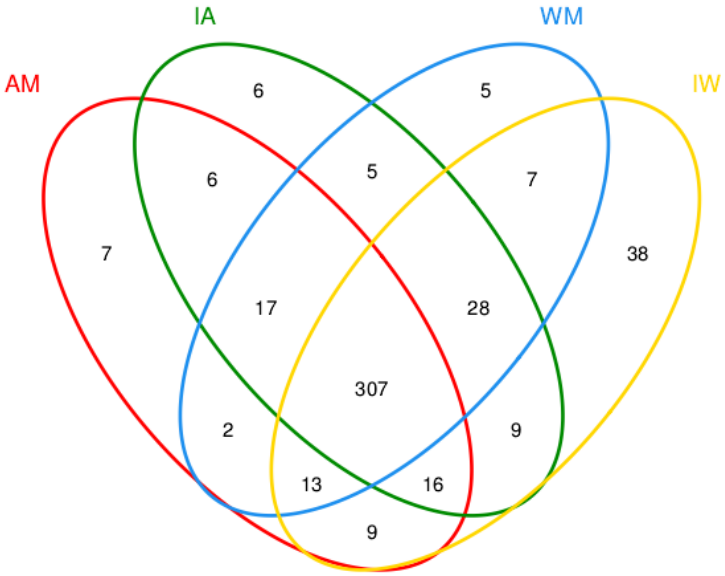

Figure 5. *Venn diagram showing the shared bacterial OTUs (at a distance of 0.03) in 4 soil samples. Alfalfa monoculture (AM), intercropping alfalfa (IA), wheat monoculture (WM), and intercropping wheat (IW)*

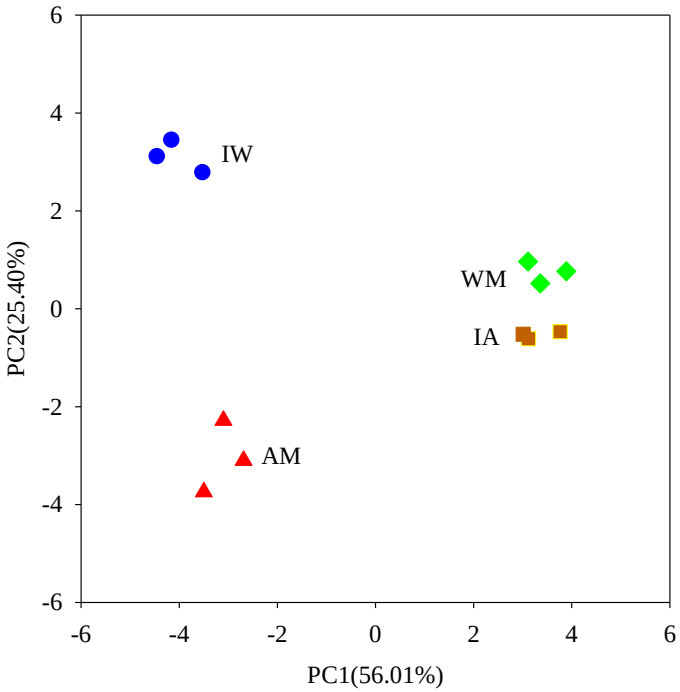

Figure 6. *Principal component analysis (PCA) of bacterial communities from Alfalfa monoculture (AM), intercropping alfalfa (IA), wheat monoculture (WM), and intercropping wheat (IW) based on pyrosequencing of the 16S rDNA gene. PCA were generated using the presence of each OTU (at a distance level of 3%) found in each clone library. Principal components (PCs) 1 and 2 explained 56.01% and 25.40% of the variance, respectively*

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Discussion

In this study, 16S rDNA gene clone library analyses were undertaken to study soil bacterial communities in wheat-alfalfa intercropping systems. A single study refers to cereal-legume intercropping, dominantly focusing on soil cultivable microbial flora (Chai et al., 2004). But data on overall bacterial communities living in cereal and legume roots vicinity are still lacking. To our knowledge, this study is the first to report data concerning the uncultivable microbial flora surrounding wheat and alfalfa in monoculture and intercropping systems, respectively. It is known that a wide range of factors influences soil bacterial communities. Soil type, plant species and cropping patterns are the reasons that most affect the bacterial communities in soils (Igwe Vannette, 2019; Rui et al., 2015). Shannon diversity analyses revealed a richer bacterial community in intercropping soil than that of monoculture (*Table 1*). This observation may be supported by PCA result that demonstrates that soil bacterial communities obtained from monoculture and intercropping system were different, regardless of plant species (*Fig. 6*). The result demonstrated bacterial communities in rhizosphere soils were indeed affected by intercropping patterns. The presence of alfalfa plants contributed to attenuate eventual bacterial community variations occurring in intercropping wheat. Indeed, legume-based intercropping systems present a more heterogeneous vegetation cover, a patchier distribution of plant litter and rooting patterns that can affect soil properties and microbial communities (Lacombe et al., 2009; Reynolds et al., 2007). The result demonstrated bacterial communities in rhizosphere soils were indeed affected by intercropping patterns. Moreover , an influence of plant species on bacterial diversity was observed.

The high diversity of soil bacteria in intercropping and monoculture systems were shown based on the sequence analyses. For microbial analysis of soil, the dominant taxonomic groups were *Proteobacteria*, *Acidobacteria*, *Bacteroidetes*, *Actinobacteria*, *Planctomycetes*, *Chloroflexi*, and *Nitrospirae* (*Fig. A2*). These phyla have been described as common inhabitants of farmland soils (Li et al., 2016, 2020). The diversity of *Actinobacteria* and *Bacteroidetes* were relatively abundant in 4 libraries and present at higher percentages in intercropping libraries. Additionally, some phylum that were not abundant in these libraries, including *Gemmatimonadetes* and *Bacteria*_unclassified, showed higher percentages in intercropping alfalfa soils ($P < 0.05$), which indicated an important role of intercropping in shaping the soil microbial communities. Other phylotypes showed the opposite variation in monoculture and intercropping system. The diversity of *Acidobacteria* and *Nitrospirae* were relatively abundant in 4 libraries and present at higher percentages in monoculture wheat libraries ($P < 0.05$). In addition, *Firmicutes* showed higher percentages in monoculture alfalfa soils. There were another two different changes including bacterial populations absent or appeared only in monoculture libraries, whereas the phylotypes of *Syntrophobacterales* and *Hydrogenophilale* appeared only in intercropping libraries.

Most of previous studies have indicated that legume and cereal intercropping is profitable for increasing crop yield (Li et al., 2007). In our research, the yield data clearly demonstrates the superiority of the use of intercropping pattern (*Fig. 1*). This beneficial effect may be attributed to the maintenance and improvement of microbial activity and community composition (Fu et al., 2018). Two main mechanisms that soil microbes affect plant productivity can be distinguished: direct effects on plants by means of root-associated organisms that form mutualistic or pathogenic relationships with plants, and indirect effects by means of the action of free-living microbes that

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change rates of nutrient supply and the partitioning of resources (Van Der Heijden et al., 2008). In our study, the complex changes of bacterial community diversity and abundant identified in monoculture and intercropping libraries raises one question. What relationship did the changing bacterial population and increasing of crop yield in intercropping pattern? Soil nutrition (eg. nitrogen, phosphorus and potassium or some other non-N nutrient that is limiting in a habitat) limits plant productivity (Chapin III, 1980), which showed plant-soil feedback processes are also dominating. Beneficial plant rhizobacteria are associated with the surfaces of plant roots and may increase plant yield via mechanisms that improved mineral nutrient uptake, disease suppression, or phytohormone production (Sameh and Youseif, 2018; Sood et al., 2018; Hokkanen and Lynch, 1995). Legumes and nonlegumes can "complement" each other in the use of N sources since both the legume and nonlegume utilize soil inorganic N sources, but nodule in leguminous plants can also fix atmospheric N_2 in symbiosis with *Rhizobiales* (Jensen, 1996a, b). Wheat intercropping libraries had more *Rhizobiales* than wheat monoculture libraries (*Fig. 2*). The amount of soil nitrogen-fixing bacteria in alfalfa intercropping soils increased mainly in the presence of wheat. Previous studies also confirm that N_2 fixation of legumes may be improved by intercropping when the no legume is a strong competitor for soil inorganic N (Giller et al., 1991; Karpenstein-Machan et al., 2000; Hauggaard-Nielsen et al., 2001).

The recent studies of overyielding in agriculture intercropping systems found a important mechanism underlying such facilitation is the ability of some crop species to chemically mobilize otherwise-unavailable forms of one or more limiting soil nutrients such as phosphorus (Li et al., 2014). Plant do not take up organic P directly, rather, organic P is first hydrolyzed by microbial or root-related phosphatases. Therefore, phosphate solubilizing bacteria have a important role in soils with low concentration of available phosphorus. Phosphate solubilizing bacteria were present in different proportions in monoculture and intercropping soils. *Burkholderiales* and *Pseudomonadales* were more abundance in intercropping soils than that monoculture soils. These bacteria can improve solubilization of fixed soil phosphorus and applied phosphates resulting in higher crop yields (Nautiyal, 1999). Moreover, *Actinobacteria* have a critical role in decomposition of soil organic materials, such as cellulose and chitin (Sykes et al., 1973). *Actinobacteria* of intercropping soils were higher than that of monoculture soils, which may be due to the presence of more organic matter used by plants in intercropping soils. Compared with monoculture, the number of organic acid in the roots exudates was increased in wheat/maize intercropping (Hao et al., 2003), which might affect some acid-sensitive microbes. *Acidobacteria* of intercropping alfalfa soils were higher than that of monouculture soils, which may be due to the presence of more organic matter used by plants in intercropping soils. *Acidobacteria* are capable of degradation of plant litter in soils (Eichorst et al., 2011), the presence of wheat debris in monoculture samples and alfalfa root exudates and litter in intercropping samples may have contributed to the observed differences.

Another route by which soil microbes affect plant productivity is disease suppression, as an example through the production of antifungal metabolites (Weller et al., 2002). Plants release enormous of chemicals through their roots, at a significant carbon cost, to combat pathogenic microorganisms and attract beneficial ones (Badri et al., 2009). The activity and effects of beneficial rhizosphere microorganisms on plant growth and health are well documented for bacteria like by *Pseudomonales* and *Burkholdera* (Badri et al., 2009). *Pseudomonales* and *Burkholderiales* were present at

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higher relatively abundances in intercropping alfalfa and wheat, respectively. These bacteria protect several major agricultural crops against disease phenomenon that is likely to be also important in natural ecosystems (Van Der Heijden et al., 2008). Moreover, it is reported *Bacillus* also was major antagonists (Yuan et al., 2016), which can promote plant growth, protect against fungal pathogen attack (Asaka and Shoda, 1996), and play a role in the degradation of organic polymers in the soil (Emmert and Handelsman, 1999). Similar as *Pseudomonales*, *Bacillus* was present at higher relatively abundances in intercropping alfalfa. There are four main groups of plant pathogens, but only fungi and nematodes are major players in the soil (Hilbig and Allen, 2019). The structure and function of other plant pathogens also need further discovery. Consequently, the analyses of bacterial communities in our study can provided general information about the relationships between soil bacterial communities and intercropping patterns. Many of bacterial populations identified in 4 libraries showed significant differences between monoculture and intercropping systems, but only a few have been reported as being able to improve plant productivity and the functions of other populations also still await discovery.

Conclusion

Intercropping pattern improve crop productivity in the field. Our findings indicated that intercropping is a determinant in shaping bacterial community in soils. Intercropping led to variations in the plant-growth promoting rhizobactertia in the rhizosphere of wheat and alfalfa. The resulting microbial community is likely to be a mixture of the species-specific. The results provide a strong evidence for improving the microbial diversity of rhizosphere soil and the nutrients of rhizosphere soil in wheat/alfalfa intercropping, and provide a direction for further research on the role of specific microorganisms. The reason of the yield advantage of intercropping system caused by the change of crop rhizosphere microbial community structure needs further study.

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APPENDIX

Table A1. *Number of 16S rDNA gene sequences derived from 4 libraries*

Sample	No. obtained sequences ≥ 400 bp	Bases (bp)	Average length (bp)
Monoculture alfalfa	10405	4123641	396.31
Alfalfa intercropping	14044	5565166	396.27
Monoculture wheat	13043	5165858	396.06
Wheat intercropping	11902	4716160	396.25

Figure A1. *Rarefaction curves indicating the observed number of OTUs at 3% genetic distances in 4 libraries. Alfalfa monoculture (AM), intercropping alfalfa (IA), wheat monoculture (WM), and intercropping wheat (IW)*

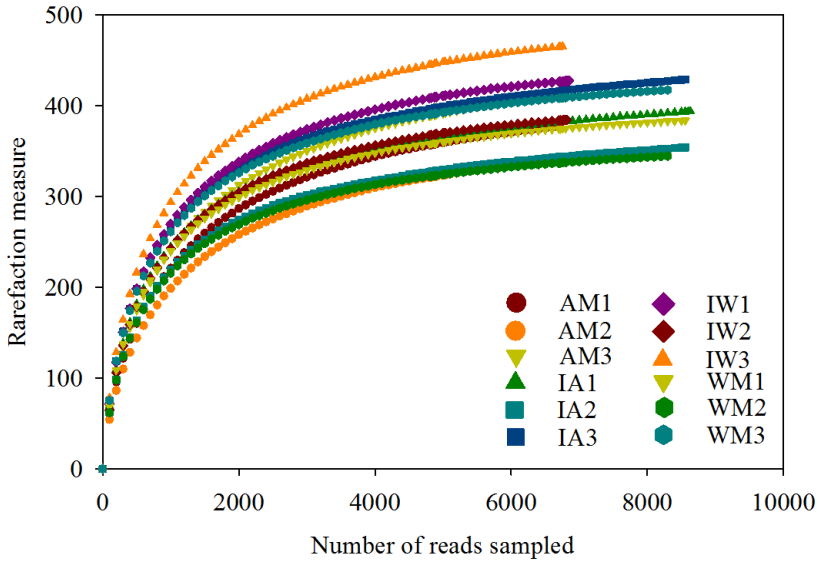
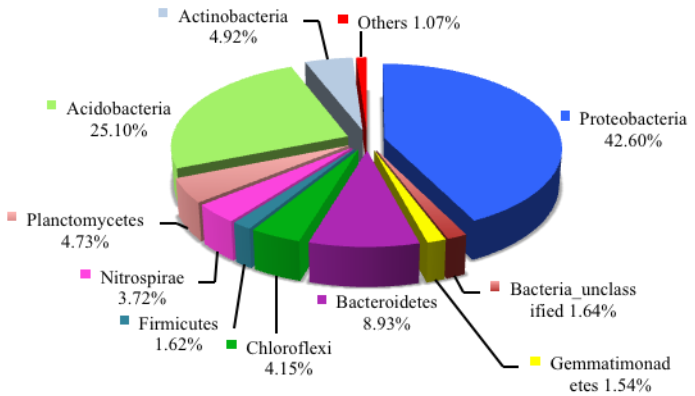


Figure A2. *Composition of bacterial taxonomic groups*



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