

Scotland's Rural College

## **Bacterial communities in paddy soils changed by milk vetch as green manure**

Gao, Songjuan; Cao, Weidong; Zhou, Guopeng; Rees, Robert M.

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# 1 **Bacterial communities in paddy soils changed by milk vetch as green** 2 **manure: A study conducted across six provinces in south China**

## 3 4 ABSTRACT

5 The use of green manures contributes to sustainable soil and nutrient management in  
6 agriculture, but the response of soil microbial communities to different fertilization regimes at  
7 regional scale are more uncertain. A study was undertaken across multiple sites and years in  
8 Hunan, Jiangxi, Anhui, Henan, Hubei and Fujian provinces in south China to investigate the  
9 effects of green manuring on the community structure and function of soil bacterial  
10 communities in rice-green manure cropping systems. The study included four treatments; (i)  
11 winter fallow with no chemical fertilizer as a control (NF), (ii) milk vetch as green manure  
12 without chemical fertilizer (GM), (iii) winter fallow and chemical fertilizer (CF), and (iv) a  
13 combination of chemical fertilizer and milk vetch (GMCF). Significant differences were  
14 found in the responses of soil microbial communities at different sites, the sampling sites  
15 explained 72.33% ( $F = 36.59$ ,  $P = 0.001$ ) of the community composition. The bacterial  
16 communities from Anhui, Henan and Hubei were broadly similar, while those in Hunan were  
17 distinctly different from other locations. The analysis of Weighted UniFrac distances showed  
18 that milk vetch changed soil microbial communities compared with winter fallow.  
19 Proteobacteria and Chloroflexi predominate in these paddy soils, but the application of green  
20 manures increased the relative abundance of Actinobacteria. There is evidence to show that  
21 the functional microbes which play important roles in the cycling of soil carbon, nitrogen and  
22 sulfur were changed after several years of milk vetch utilization (LDA score  $> 2$ ). The  
23 abundance of methane-oxidizing bacteria and sulfur-reducing bacteria increased, and the  
24 microbes involved in nitrogen fixation, nitrification and denitrification also increased in some  
25 provinces. We concluded that the application of milk vetch changed the bacterial community  
26 structure and has impacts on the functional groups related to nutrient transformation in soils at  
27 a regional scale.

28 *Key Words:* milk vetch; soil fertility; bacterial communities; paddy soil; functional groups

## 29 30 INTRODUCTION

31 The application of green manures in rice cropping system is known to be an effective  
32 practice for maintaining high yields of rice while at the same time providing a wide range of  
33 environmental benefits (Couëdel *et al.*, 2018; Gao *et al.*, 2013; Xie *et al.*, 2016). Milk vetch  
34 (*Astragalus sinicus* L.) is widely used in south China as winter green manure. Previous  
35 studies have shown that the utilization of milk vetch can improve the productivity and  
36 sustainability of paddy soils (Hwang *et al.*, 2015; Xie *et al.*, 2016). The incorporation of green  
37 manures changes microbial processes, thereby affecting microbial community structures and

38 nutrient cycling, especially soil C, N, S, etc. (Gao *et al.*, 2015; Wang *et al.*, 2013). However,  
39 the nature of these changes and their consequences for nutrient cycling are poorly understood.

40 Soil microorganisms play pivotal roles in nutrient cycling processes in agro-ecosystems,  
41 and changes in the microbial communities can be indicator of soil environment and nutrient  
42 turnover. The type and chemical composition of organic substrates affect the diversity,  
43 composition and functional group of soil microbes (Pan *et al.*, 2016). Climatic conditions  
44 (Guo *et al.*, 2015; Zhang *et al.*, 2013), cropping systems (Zhao *et al.*, 2014), field  
45 management practices and fertilization regimes (Lagomarsino *et al.*, 2016; Zhang *et al.*,  
46 2017b; Zhao *et al.*, 2016) can also interact with organic matter addition to affect the  
47 composition and function of soil microbes. Previous studies reported that the use of mineral  
48 fertilizers may cause a decrease in soil microbial diversity, resulting in a decline in soil  
49 fertility (Schmid *et al.*, 2018; Zhang *et al.*, 2017c). Most organic soil amendments will  
50 introduce complex organic substrates into soil, which are metabolized by specialized  
51 microorganisms, thus this would be expected to cause a change of community composition  
52 and functional groups (Schmid *et al.*, 2018). The long-term combined utilization of organic  
53 and inorganic fertilizers also therefore increases soil fertility and bacterial community  
54 structure with implications for the decomposition of native soil organic matter (Li *et al.*,  
55 2017).

56 The incorporation of green manures introduces large amounts of fresh organic matter into  
57 soils, which may cause dramatic changes in soil nutrient transformations and associated  
58 microorganisms. A distinguishing feature of green manures in contrast to other organic  
59 material is that their growth stage is also crucial in influencing soil processes. Zhang *et al.*,  
60 (2017b) showed that 31 years' utilization of different green manures in red paddy soil shaped  
61 the microbial communities of rice rhizosphere. Other studies revealed that utilization of green  
62 manure can improve soil microbial characteristics, including increase soil microbial biomass  
63 and soil extracellular enzyme activity (Elfstrand *et al.*, 2007a; Ye *et al.*, 2014). The changes in  
64 the soil microbial communities may partly explain the mechanisms by which green manures  
65 improve soil fertility and increase grain yields. But many uncertainties remain regarding the  
66 relationship between organic amendments and specific changes to individual microbial  
67 groups, especially at large regional scales. The response of microbial communities at different  
68 sites with varying soil conditions and climate are particularly difficult to predict. A network of  
69 experiments in six provinces was therefore established to investigate the effects of green  
70 manure on soil properties and microbial communities in different paddy soils, and to explore  
71 the differences and relationships between different sites. The hypotheses were: (i) the soil  
72 bacterial communities would be changed by 4 or 7 years application of milk vetch and  
73 differed between sites, and (ii) some particular bacterial groups can be related to milk vetch  
74 application.

## 75 MATERIALS AND METHODS

### 76 *Field description and experiment design*

77 The study sites were located at experimental stations in six provinces in south China  
78 (Table 1). All sites were located on paddy soils, classified as stagnant Anthrosols (FAO,  
79 2015). The experiments were set up in a completely randomized block design with three

80 (Hunan, Jiangxi, Hubei, Fujian and Anhui) or four (Henan) replicates of each treatment in  
 81 2008 (Hunan, Jiangxi, Henan, Hubei, Fujian) and 2011 (Anhui). Each plot was 20 m<sup>2</sup> in area  
 82 (4 m×5 m), and the four treatments were; (i) winter fallow and no chemical fertilizer as  
 83 control (NF), (ii) milk vetch as green manure (GM), (iii) winter fallow and chemical fertilizer  
 84 (CF), and (iv) a combination of chemical fertilizer and milk vetch (GMCF). Different rates of  
 85 chemical fertilizer, which are based on local farmer practices were used at the different sites  
 86 (Table 2). The incorporation rate of milk vetch was 22500 kg (FW) ha<sup>-1</sup> each year. The  
 87 management of the field experiments were described in our previous study (van  
 88 Maarschalkerweerd *et al.*, 2016). The cropping rotation in Henan and Fujian was single rice-  
 89 green manure, and in Hunan, Jiangxi, Anhui, and in Hubei it was double rice-green manure.

90

91 TABLE 1. The location and climate of different experimental sites.

Site	Longitude (°E)	Latitude (°N)	Mean annual temperature (°C)	Precipitation (mm)	Start year
Hunan	112.39	29.37	16.6	1776	2008
Jiangxi	115.07	28.19	15.3~17.7	1936	2008
Anhui	117.48	30.66	16.1	1400-1700	2011
Henan	114.08	32.12	15.1~15.3	1900~2100	2008
Hubei	113.08	29.23	15.5	1755	2008
Fujian	119.03	26.08	20~25	1700~1980	2008

92

93 TABLE 2. Fertilizer application rates at the six sites.

Site	Fertilizer amount of early rice (kg ha <sup>-1</sup> )			Fertilizer amount of late rice (kg ha <sup>-1</sup> )		
	N	P <sub>2</sub> O <sub>5</sub>	K <sub>2</sub> O	N	P <sub>2</sub> O <sub>5</sub>	K <sub>2</sub> O
Hunan	150	75	120	150	75	120
Jiangxi	150	75	120	180	75	150
Anhui	165	75	90	180	75	90
Henan	225	135	135	—	—	—
Hubei	180	90	105	180	90	105
Fujian	135	54	94.5	—	—	—

94 N, P and K fertilizers are urea, calcium superphosphate, and potassium chloride, respectively.

95

### 96 *Soil sampling*

97 All the soils at the six sites were sampled after the harvest of late rice or single rice in  
 98 November or December of 2014. The soil samples were collected from five points in each  
 99 plot, and the top-soil (0-20 cm) was sampled. Plant residues and stones in collected soil  
 100 samples were removed, then the samples were mixed well and sieved (< 2 mm). Parts of the  
 101 soil sample were stored at -80°C for molecular analysis, and the others were stored at 4°C or  
 102 air dried for the physical and chemical analysis.

103

### 104 *Chemical analysis*

105 Soil physical and chemical analyses were conducted according to the methods described  
106 by Lu (2000). Soil pH was tested using pH meter with a soil to water ratio of 1:2.5 (m:v). Soil  
107 organic matter (SOM) was tested using potassium dichromate oxidation method. Soil total N  
108 (TN) was measured using the Kjeldahl method. Available phosphorus (AP) was extracted by  
109 0.5 mol L<sup>-1</sup> NaHCO<sub>3</sub> and available potassium (AK) were extracted by 1 mol L<sup>-1</sup>  
110 CH<sub>3</sub>COONH<sub>4</sub>. Soil mineral nitrogen (NH<sub>4</sub><sup>+</sup>-N, and NO<sub>3</sub><sup>-</sup>-N) was extracted using 2 mol L<sup>-1</sup>  
111 KCl, and then determined using a continuous flow analyzer (AA3, SEAL, Germany). Soil  
112 texture was measured using pipette method, which following Stoke's law. The results of  
113 chemical properties and the soil texture are listed in Tables A.1 and A.2.

114

#### 115 *DNA extraction and sequencing*

116 Soil DNA was extracted using the FastDNA Spin Kit for Soil (MP Bio, Santa Ana, CA,  
117 USA). The extracted DNA samples were quantified using a Nanodrop 2000 spectrophotometer  
118 (Thermo Fisher, Waltham, MA, USA) and then stored at -80°C for further analysis. The  
119 primers of 16S rDNA sequence were 338F/806R (ACTCCTACGGGAGGCAGCA /  
120 GGACTACHVGGGTWTCTAAT) (Chu *et al.*, 2015). The length of the primer was 468 bp.  
121 Barcode oligonucleotides were ligated to primer to distinguish the amplicons from different  
122 samples. PCR amplification was conducted using a GeneAmp PCR System 9700 (Life  
123 Technologies, Carlsbad, CA, USA). Then the purified PCR products were quantified and  
124 pooled at equal concentration. Sequencing of the PCR amplicon was performed on the  
125 Illumina MiSeq PE300 platform (Caporaso *et al.*, 2012).

126

#### 127 *Bioinformatics and statistical analyses*

128 The sequences obtained were trimmed and screened using Trimmomatic and FLASH  
129 (version 1.2.7, <http://ccb.jhu.edu/software/FLASH/>). Briefly, according to their unique  
130 barcodes, sequencing reads were assigned to each sample, then low-quality sequences  
131 (average quality score lower than 20, and length less than 50 bp) were removed. The  
132 remaining reads were then spliced based on the overlap sequence (length > 10 bp), and no  
133 ambiguous base was permitted in the overlap region. The same number of sequences in each  
134 pyrosequencing library was subsampled randomly by Mothur software (Schloss *et al.*, 2009)  
135 ([http://www.mothur.org/wiki/Schloss\\_SOP](http://www.mothur.org/wiki/Schloss_SOP)) to eliminate the bias of libraries' alpha diversity  
136 comparison, based on the minimum reads number of all the samples (each of 23650 reads,  
137 after quality control).

138 The remaining high-quality reads were then aligned and clustered into operational  
139 taxonomic units (OTUs) using Usearch (version 7.1 <http://drive5.com/uparse/>) (Edgar, 2013).  
140 The sequence identity threshold is 97%. The fastx\_unique command was used to remove the  
141 duplicated sequences, and the sortbysize derep.fasta command was used to discard singletons.  
142 The representative sequences of the OTUs were compared to the Sliva database using RDA  
143 classifier (Wang *et al.*, 2007) and then get the taxa of each sample. OTUs number, Good's  
144 coverage (Good, 1953), Chao 1 richness (Chao, 1984) and the Shannon index (Chao and  
145 Shen, 2003) were calculated to estimate the alpha diversity of the samples. The

146 approximately-maximum likelihood phylogenetic trees were conducted using FastTree  
147 (version 2.1.3, <http://www.microbesonline.org/fasttree/>). Then the Weighted UniFrac  
148 distances were calculated using Fastunifrac (Hamady *et al.*, 2010)  
149 (<http://unifrac.colorado.edu/>). The heatmaps based on the Weighted UniFrac distances and the  
150 principle coordinate analysis (PCoA) based on Bray-Curtis distances were analysed in R (R  
151 development Core Team, 2008). Linear discriminant analysis (LDA) effect size (LEfSe) was  
152 employed to identify the specific phylotypes responding to the green manure treatments. A  
153 non-parametric factorial Kruskal-Wallis (KW) sum-rank test was performed to estimate the  
154 differences between green manure and winter fallow treatments and finding out the  
155 phylotypes with significant differences. The groups with logarithmic LDA scores larger than 2  
156 were evaluated as significantly different, and cladogram was constructed using the LDA  
157 score. A nonparametric multivariate analysis of variance (Adonis) was conducted using the  
158 “vegan” package in R, and a mantel test using PASSaGE software.

159 The pyrosequencing data were deposited into the NCBI Sequence Read Archive (SRA)  
160 database, and the accession number is SRP140602.

161

## 162 RESULTS

### 163 *Alpha diversity*

164 Good’s coverage of all the samples were higher than 95% (Table A.3), suggesting that  
165 the bacterial communities could be well reflected using the obtained libraries. OTU numbers,  
166 Chao 1 richness and the Shannon index were selected to represent the alpha diversity of soil  
167 bacterial communities (Table A.4). The OTU number, Chao 1 richness and Shannon index  
168 differed among sites, and the alpha diversity was highest in Anhui and lowest in Jiangxi (Fig  
169 1). The OTU numbers of all the samples were ranged from 1690 to 3493. Compared with NF,  
170 the GM and GMCF treatments increased OTU numbers and the Shannon index ( $P<0.05$ ) and  
171 the GM treatment increased Chao 1 richness in Jiangxi ( $P<0.05$ ). In OTU numbers, Chao 1  
172 richness and the Shannon index, there was no significant difference at other sites between  
173 winter fallow and green manure treatments (Table A.4).

174

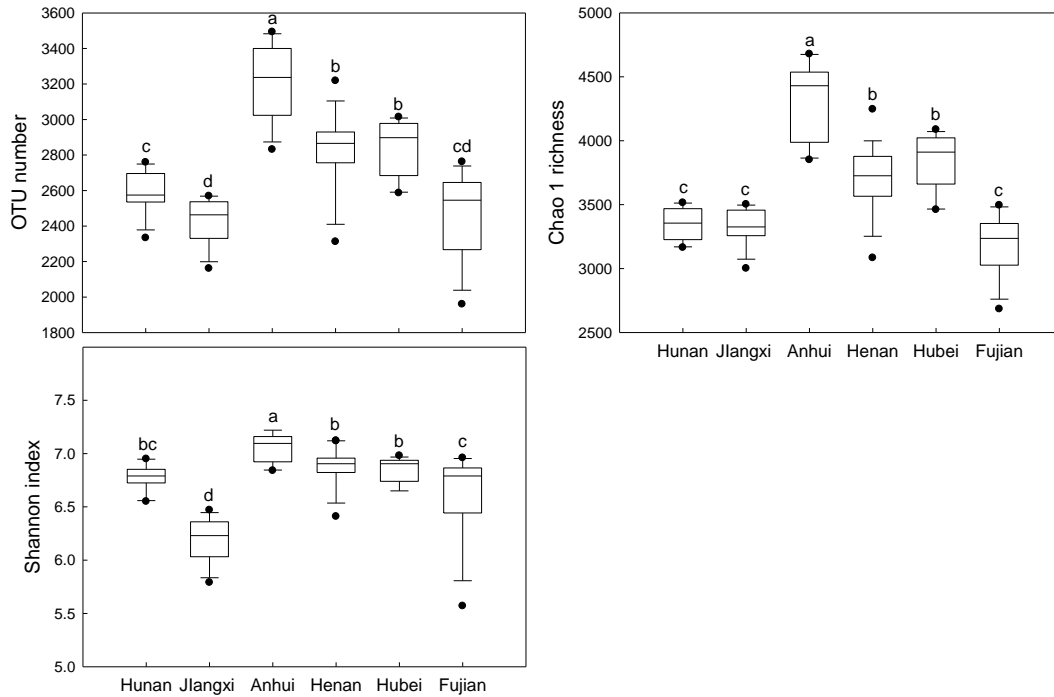


Fig. 1. The OTU number, Chao 1 richness and Shannon index at the six sites

### Bacterial composition

In total 10 phyla (relative abundance > 1%) were chosen to evaluate bacterial composition (Fig 2). Proteobacteria were predominant in Hunan, Jiangxi, Anhui, Henan and Hubei, and the relative abundances were 30.10%, 28.30%, 30.76%, 27.95% and 27.51%, respectively. This was followed by the Chloroflexi (the relative abundances were 30.06%, 22.41%, 20.70%, 25.09% and 16.51%, respectively) (Fig 2). In Fujian, the relative abundance of Chloroflexi (31.75%) was higher than Proteobacteria (22.52%) (Fig 2). The two-way ANOVA (Table 3) showed that sampling site and the interaction between site and treatment had significant effects on the abundances of Proteobacteria and Chloroflexi. The abundances of Acidobacteria ranged from 5.29% to 29.12%, and were affected significantly by both sampling site and green manure treatment (Fig 2 and Table 3). Treatments with winter green manuring increased the abundances of Actinobacteria in Jiangxi, Henan and Fujian provinces, and decreased the abundance of Nitrospirae in Hunan compared with the winter fallow (Fig 2 and Table 3). The relative abundances of Nitrospirae ranged from 2.11% to 15.74%, although this varied between sites. In Hunan, Jiangxi, Anhui and Henan provinces, the relative abundances of Firmicutes were higher in the treatments with milk vetch than the winter fallow treatments (Fig 2 and Table 3), suggesting that the abundances of Firmicutes were also significantly affected by green manuring treatment. The relative abundances of Bacteroidetes and Gemmatimonadetes ranged from 0.21% to 4.52% and from 0.69% to 2.98%, respectively, and clear differences were found between sites (Fig 2 and Table 3). The relative abundances of Chlorobi and Planctomycetes ranged from 0.35% to 3.72% and from 0.13% to 5.63%, respectively. Compared with winter fallow, the relative abundance of Chlorobi in Jiangxi

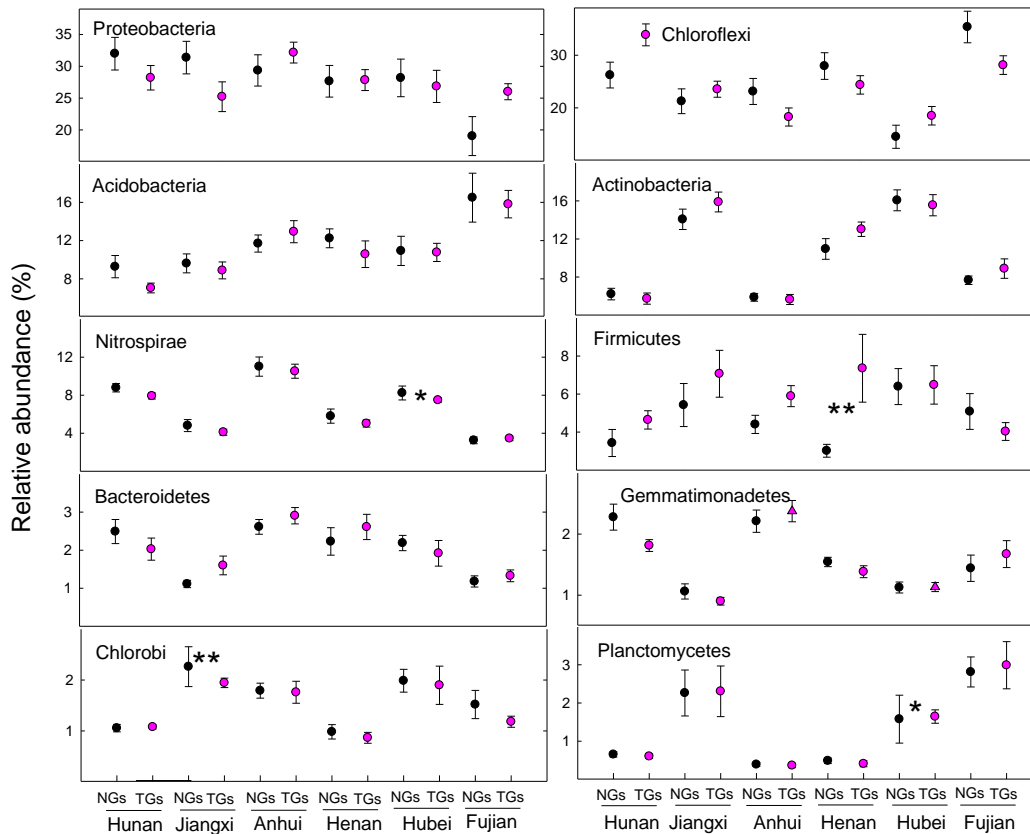
200 under the milk vetch treatment decreased, while the relative abundance of Planctomycetes in  
 201 Hubei increased (Fig 2).

202

203 TABLE 3. The effects of site and green manure treatment on the relative abundances of major phylum  
 204 (>1%) and their interaction effects

	Proteoba cteria	Chloro flexi	Acidob acteria	Actinob acteria	Nitrospi rae	Firmic utes	Bacter oidetes	Gemmatim onadetes	Chloro bi	Planctom ycetes
Site	<b>2.86*</b>	<b>13.72**</b>	<b>8.47**</b>	<b>51.86**</b>	<b>43.17**</b>	1.65	<b>8.72**</b>	<b>23.83**</b>	<b>11.61**</b>	<b>16.65**</b>
Treatment	0.25	0.89	1.15	<b>3.42*</b>	1.07	<b>2.97*</b>	0.21	0.66	0.79	0.21
Site*Treat ment	1.33	<b>1.93*</b>	0.67	0.59	0.55	1.25	0.66	0.82	0.44	0.64

205 Note: Values in the table is the F value obtained from two-way ANOVA. \* represent significant  
 206 difference at  $P<0.05$  level between groups, \*\* represent significant difference at  $P<0.01$  level between  
 207 groups.



208

209 Fig. 2. The relative abundances of major phyla (>1%) with or without winter green manure at different  
 210 sites.

211 Note: NGs indicates NF (winter fallow and no chemical fertilizer as control) and CF (winter fallow and  
 212 chemical fertilizer); TGs indicates GM (milk vetch as green manure) and GMCF (the combination of  
 213 chemical fertilizer and milk vetch); \* represent significant difference at  $P<0.05$  level between groups,  
 214 \*\* represent significant difference at  $P<0.01$  level between groups.



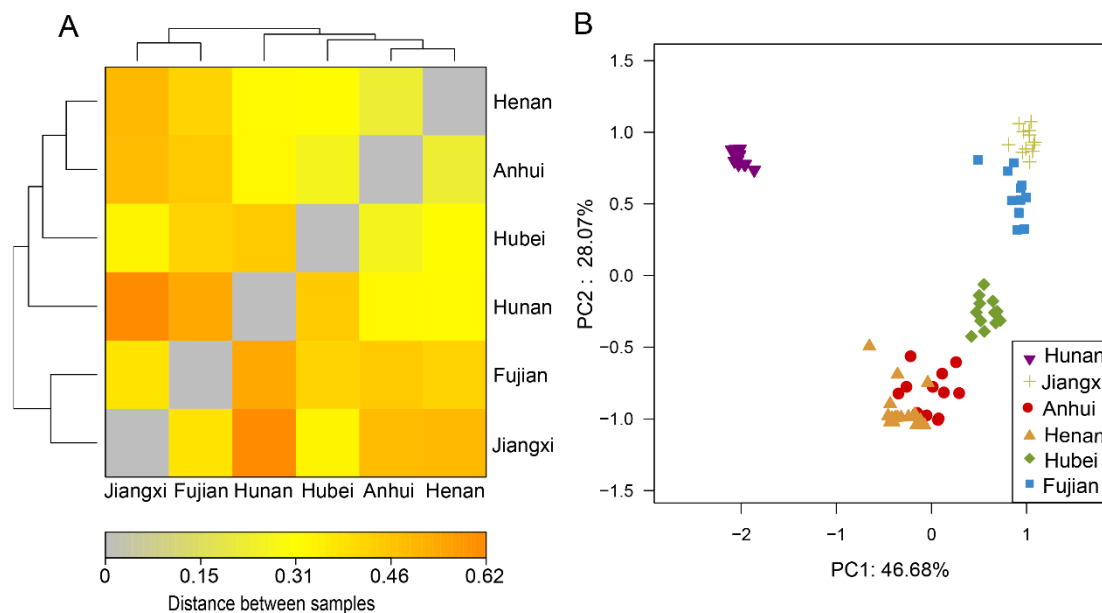
215

## 216 *Community difference*

217 Weighted UniFrac distances were calculated to evaluate the community differences  
218 between sites and treatments. In Hunan, Henan and Fujian provinces, the distances between  
219 NF and GMCF were largest, while the largest distances occurred between NF and GM  
220 treatments in Jiangxi, Anhui and Hubei provinces (Fig A.1). Results of the Adonis analysis  
221 showed that the green manure treatment explained 35.68% ( $F = 36.53$ ,  $P = 0.028$ ) of the  
222 microflora variations in Hunan province, and explained 35.30% ( $F = 1.45$ ,  $P = 0.058$ ) and  
223 24.12% ( $F = 1.27$ ,  $P = 0.074$ ) of the community variations in Jiangxi and Henan provinces,  
224 respectively.

225 The community differences between sites were evaluated using both Weighted UniFrac  
226 distances (Fig 3A) and Bray-Curtis distances (Fig 3B). The Weighted UniFrac distances  
227 showed that there was little difference between microbial communities in Anhui, Henan and  
228 Hubei, and the samples of Hunan were far away from other sites (Fig 3A). The principal co-  
229 ordinates analysis (PCoA) based on the Bray-Curtis distances showed similar results (Fig 3B).  
230 The samples of Hunan were separately in the second quadrant, and Jiangxi and Fujian were  
231 clustered together in the first quadrant, and the other three sites were clustered in the third and  
232 fourth quadrant (Fig 3B). The results of Adonis showed that sampling sites explained 72.33%  
233 ( $F = 36.59$ ,  $P = 0.001$ ) of the community variations.

234



235

236 Fig 3 The Weighted UniFrac distance (A) and principal co-ordinates analysis (PCoA) based on the  
237 Bray-Curtis distance among sites (B).

238 Note: The darker yellow color represents the bigger distance between treatments.

239

## 240 *Effects of milk vetch on functional genus*

241 The LDA effects size analysis was conducted to find out the effects of green manure  
 242 utilization on soil bacteria. The cladogram showed that both the number and species of  
 243 enriched groups changed between sites (Fig A.2). At the six sites, the most enriched groups  
 244 affected by green manure treatment were found in Anhui and in Hubei, respectively (Fig A.2).  
 245 We further investigated the functional groups involving soil carbon, nitrogen and sulfur  
 246 transformations, and found that winter green manuring enriched some of these functional  
 247 groups (Table 4). In Hunan, incorporation of milk vetch increased the abundance of  
 248 *Methylocystis* (Type II Methanotrophs), while in Anhui and Hubei, Type I methanotrophs  
 249 (*Methylobacterium*, *Methylococcus*, *Methylomonas* and *Methylosarcina* in Anhui and  
 250 *Methylocaldum* in Hubei) were significantly enhanced by green manuring. The nitrogen-  
 251 fixing bacteria *Bradyrhizobium* and *Rhizobium* were enhanced by the milk vetch treatment in  
 252 Jiangxi. *Nitrospira*, which contributes to the nitrite oxidization process, was enhanced by  
 253 winter green manuring in Hunan and Anhui. The denitrifier *Thiobacillus*, *Bacillus* and  
 254 *Hyphomicrobium* were significantly enhanced by utilization of milk vetch in Jiangxi, Henan,  
 255 and Hubei. Sulfur-reducing bacteria were also enhanced by the utilization of milk vetch.  
 256 Among them, *Desulfovibrionaceae*, *Desulfovibrio* and *Desulfobacca* in Anhui,  
 257 *Desulfitobacterium* and *Desulfosporosinus* in Henan and *Desulfobacca* in Fujian were  
 258 increased after application of milk vetch, respectively.

259

260 TABLE 4. The functional groups of bacteria enriched by milk vetch and their roles in soil nutrient  
 261 transformations in the six sites based on the results of LDA effect size analysis (LDA score > 2).

Site	Enriched functional group	Function
Hunan	<i>Nitrospira</i>	Nitrification
	<i>Methylocystis</i>	Methane oxidation
Jiangxi	<i>Bradyrhizobium</i> , <i>Rhizobium</i>	N fixation
	<i>Thiobacillus</i>	Denitrification
Anhui	<i>Methylobacterium</i> , <i>Methylococcus</i> , <i>Methylomonas</i> , <i>Methylosarcina</i>	Methane oxidation
	<i>Nitrospira</i>	Nitrification
	<i>Desulfovibrionaceae</i> , <i>Desulfovibrio</i> , <i>Desulfobacca</i>	Sulfate reduction
Henan	<i>Bacillus</i>	Denitrification
	<i>Desulfitobacterium</i> , <i>Desulfosporosinus</i>	Sulfate reduction
Hubei	<i>Methylocaldum</i>	Methane oxidation
	<i>Hyphomicrobium</i>	Denitrification
Fujian	<i>Desulfobacca</i>	Sulfate reduction

262

### 263 *Correlations between soil properties and bacterial communities*

264 Mantel tests were conducted to evaluate the correlations between soil properties and  
 265 bacterial communities (Table 5). In Hunan, soil microbial communities showed significant  
 266 correlations with soil organic matter ( $P = 0.011$ ) and soil available K ( $P = 0.013$ ). In Anhui,  
 267 soil pH and bacterial communities were significantly correlated ( $P = 0.044$ ). No significant

268 correlation was found in other sites.

269

270 TABLE 5. Mantel test of the relationship between soil properties and bacterial communities.

Soil properties	Hunan		Jiangxi		Anhui		Henan		Hubei		Fujian	
	r	P	r	P	r	P	r	P	r	P	r	P
pH	0.02	0.88	0.09	0.55	0.27	<b>0.04*</b>	0.09	0.46	0.15	0.35	-0.06	0.66
SOM	-0.33	<b>0.01*</b>	-0.19	0.18	-0.14	0.27	-0.07	0.65	-0.02	0.91	0.21	0.09
Total N	-0.02	0.89	-0.05	0.66	0.02	0.90	-0.09	0.53	0.12	0.94	-0.09	0.63
NH <sub>4</sub> <sup>+</sup> -N	-0.01	0.95	-0.08	0.56	0.39	<b>0.06</b>	-0.02	0.87	-0.14	0.42	-0.19	0.18
NO <sub>3</sub> <sup>-</sup> -N	-0.11	0.39	0.01	0.93	-0.11	0.54	0.02	0.92	0.02	0.89	0.23	0.24
Available P	0.20	0.11	0.20	0.12	0.06	0.62	-0.08	0.33	0.16	0.30	-0.04	0.61
Available K	0.32	<b>0.01*</b>	0.06	0.69	0.03	0.86	-0.09	0.56	0.01	0.98	-0.04	0.63

271 Note: SOM, soil organic matter. r is the correlation coefficient of mantel test. \* represent  $P < 0.05$ .

272

## 273 DISCUSSION

274 Our results suggested that the application of milk vetch in paddy soils strongly affects the  
275 diversity, composition and functional groups of soil bacteria, in ways that are broadly  
276 consistent with previously published studies (Elfstrand *et al.*, 2007a; Elfstrand *et al.*, 2007b;  
277 Gao *et al.*, 2015; Zhang *et al.*, 2017b). Changes in the composition of soil bacterial  
278 communities were found at all the six sites after milk vetch incorporation. Previous studies  
279 have reported that long-term application of manure enhanced soil microbial diversity, and  
280 changed the interactive relationship between plants and soil microorganisms (Ai *et al.*, 2015).  
281 Zhao *et al.*, (2016) demonstrated that the low amounts of straw incorporation had no obvious  
282 effect on soil microorganisms, but high amounts of straw input could significantly change soil  
283 microbial community structure. Application of straw was particularly important in stimulating  
284 the copiotrophic bacteria, and contributing to soil sustainability and productivity (Zhao *et al.*,  
285 2017). Green manure incorporation in this study also had significant impacts on soil  
286 properties and soil microorganisms. The utilization of milk vetch increased the abundances of  
287 Actinobacteria and Firmicutes in some of our study sites. Actinobacteria could promote the  
288 decomposition of plant residues, and therefore play important roles in soil nutrient cycling.  
289 The incorporation of green manures provides additional substrate for Actinobacteria and thus  
290 enhances their growth. This phenomenon was observed at more than one site, indicating a  
291 broader relevance at the regional scale. Wang *et al.*, (2015) found that the communities and  
292 functions of soil microorganisms showed a large variation that could be linked to the  
293 alternating wetting and drying of the paddy soil. The rice-winter green manure cropping  
294 system is also strongly influenced by wetting-drying cycles. However, the underlying  
295 mechanism by which changes in soil microbial communities structure take place after green  
296 manure application needs further study.

297 The soil environment and its interaction with management plays a critical role in shaping  
298 soil microbial community structure. The growth and decomposition of green manure plants  
299 had different influences on soil conditions and caused various responses of microorganism in  
300 different sites. Results showed that the community structure of soil bacteria in Hunan was

301 significantly different to that at other sites. This may be caused by the differences of soil  
302 conditions, especially soil pH. Paddy soils in Hunan are typically alkaline purple alluvial  
303 soils, which differ from the neutral or acidic paddy soils at the other five sites. Previous  
304 studies proved that soil pH is a pivotal indicator in shaping the bacterial communities  
305 (Hendriksen *et al.*, 2016; Miranda *et al.*, 2018; Osborne *et al.*, 2011). A significant correlation  
306 between soil pH and bacterial communities was found in Anhui, and demonstrated that the  
307 differences in soil pH may be one of the main factors that impact bacterial communities.  
308 Possibly because of the nearby proximity of sites, bacterial communities showed considerable  
309 similarity at Jiangxi and Fujian, and at Henan and Anhui. We have shown that the soil type  
310 and climate are important drivers influencing soil bacterial communities. Guo *et al.*, (2015)  
311 reported that the community structure of soil autotrophic bacteria could be affected by  
312 elevation, soil temperature, soil water content, soil nutrient status, vegetation type, etc. Based  
313 on five years' experiment, Zhang *et al.*, (2013) reported that climatic conditions, such as  
314 precipitation, temperature, etc. had direct effects, while soil total N and soil pH had indirect  
315 effects on bacterial communities. The synthetic action of these direct and indirect factors  
316 contributed the various responses of soil microbial communities in the different sites. The  
317 bacterial communities in Hunan and Anhui were correlated with soil pH, organic matter, and  
318 some available nutrients in our study, illustrating the interaction between soil properties and  
319 microbial communities. The availability of soil nutrients (particularly available carbon and  
320 nitrogen) can restrict the abundance of Proteobacteria with increasing soil depth (Zhang *et al.*,  
321 2017a). Other studies also shown that Proteobacteria were more abundant in a higher carbon  
322 environment (Eilers *et al.*, 2010; Fierer *et al.*, 2007; Goldfarb *et al.*, 2011) and demonstrate  
323 enormous metabolic diversity (Dong *et al.*, 2017). In this study, both the abundance of  
324 Proteobacteria and the quantity of soil carbon content in Hunan and Anhui were higher than  
325 those in other sites, indicating that soil nutrient condition was a major cause of the differences  
326 in Proteobacteria distribution.

327 The transformations of soil C, N, and S, etc. are important processes in soil nutrient  
328 cycling affecting both soil fertility and plant nutrition. Soil microbes drive the biogeochemical  
329 cycles of these elements and can have positive or negative interactions with crops (Soman *et al.*  
330 *et al.*, 2016). In this study, many bacterial genera showed significant differences in abundance  
331 between milk vetch and winter fallow treatments. Such differences would be expected to alter  
332 both the rates and pathways of soil nutrient cycling in ways that we do not yet fully  
333 understand.

334 Methane (CH<sub>4</sub>) is one of the main greenhouse gases, and paddy soil is an important  
335 source (Nazaries *et al.*, 2013). In paddy fields, CH<sub>4</sub> is produced by methanogens (a broad  
336 spectrum of heterotrophs) and consumed by methanotrophs (autotrophs), which use CH<sub>4</sub> as  
337 their only carbon and energy sources. The balance between these processes is regarded  
338 important in the mitigation of CH<sub>4</sub> emissions (Nazaries *et al.*, 2013). Methanotrophs are  
339 traditionally clustered into Type I (*Gammaproteobacteria*) and Type II (*Alphaproteobacteria*)  
340 (Choi *et al.*, 2008; Fang *et al.*, 2000; Semrau *et al.*, 1995). In our study, either Type I or Type  
341 II methanotrophs were enhanced by green manuring in Hunan, Anhui and Hubei. Some  
342 studies indicated that the quantity of methanotrophs is negatively correlated with CH<sub>4</sub>  
343 emissions (Liu *et al.*, 2017). Lee *et al.*, (2010) reported that when 10 Mg ha<sup>-1</sup> milk vetch was

344 applied as green manure, it is possible to sustain rice productivity without increasing CH<sub>4</sub>  
345 emissions. This contrasted with an NPK fertilizer treatment in a mono-rice cropping system  
346 which had higher CH<sub>4</sub> emissions. As a leguminous plant, milk vetch has a relatively low C/N  
347 ratio, and this might be one of the reasons that milk vetch induced the lower CH<sub>4</sub> emission  
348 than the other fertilizer. These findings indicate the importance of understanding underlying  
349 microbial dynamics when developing CH<sub>4</sub> mitigation strategies.

350 Because milk vetch is a legume, its effects on rhizobial populations in paddy soils  
351 deserve special attention. Nitrification and denitrification processes are controlled by nitrifiers  
352 and denitrifiers, and are critical in determining the rates of loss and turnover of soil N in  
353 cropland ecosystems (Ishii *et al.*, 2011). In this study, the genera which are crucial in N  
354 cycling (N fixation, nitrification and denitrification) were enriched by milk vetch. These  
355 results indicated that the application of milk vetch in paddy soils have profound influences on  
356 soil N transformations. Li *et al.*, (2015) reported that the combination of inorganic N fertilizer  
357 and plant residues may be an effective way to increase N use efficiency. The N transformation  
358 processes affected by green manuring may be one of the immanent causes of the improvement  
359 of N use efficiency.

360 Soil acidification is an important issue in cropland ecosystems (Guo *et al.*, 2010). The  
361 main causes of soil acidification in China are excessive N fertilizers, sulphur oxidation  
362 (following the drainage of rice paddies) and the acidic deposition containing nitric and  
363 sulfuric acids (Guo *et al.*, 2010; Larssen and Carmichael, 2000). Sulfur-reducing prokaryotes  
364 play important roles in neutralizing soil acidity within the sulfur biogeochemical cycle. In our  
365 study, sulfur-reducing bacteria were enhanced by the utilization of milk vetch. The increase of  
366 sulfur-reducing bacteria may indicate that the utilization of milk vetch in paddy soil could be  
367 a valuable tool for alleviating soil acidification.

368

## 369 CONCLUSION

370 Green manures used within rice cropping systems are able to fundamentally change the  
371 soil microbial community structures. This research has also revealed that environmental  
372 factors such as soil conditions are important in regulating the balance of microbial  
373 populations. The understanding of the relationships between changes in microbial  
374 composition and the functional consequences of such changes are still in their infancy,  
375 especially at the regional scale. In this study, we have clearly demonstrated that management  
376 interventions, especially the introduction of green manuring, have profound impacts on some  
377 of the most important microbial groups related to the biogeochemical cycling of carbon,  
378 nitrogen, and sulphur. This information will be critical to the design of new approaches aimed  
379 at mitigating greenhouse gas emissions, ensuring better nutrient use efficiency and achieving  
380 improved sustainability in rice production.

381

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