



Soil bacterial community mediates the effect of plant material on methanogenic decomposition of soil organic matter

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ABSTRACT

Input of plant material may strongly change decomposition rates of soil organic matter (SOM), i.e. causing priming effect (PE), but the underlying mechanisms are largely unknown. We found that rice straw addition in anoxic Fuyang (F) rice field soil stimulated CH₄ production from SOM at the expense of CO₂, whereas in Uruguay (U) soil it suppressed SOM degradation to CO₂ plus CH₄ (negative PE). Reciprocal inoculation experiments with non-sterile and sterile soils showed that the soils always displayed the effect of rice straw characteristic for the live microbial community rather than for the soil physicochemical properties. Pyrosequencing of 16S rRNA genes showed that bacterial communities in these soil samples were separated into two clusters (F and U). *Symbiobacterium* was abundant or dominant in microbiota from U soil, but negligible in those from F soil. Network analysis indicated that the bacterial populations involved in SOM decomposition were different between soils of F and U results; moreover, they were more tightly connected to methanogens in U than in F clusters. Ultimately, our results suggested that the PE of rice straw is mediated by the composition and activity of soil microbial community.

1. Introduction

Incorporation of plant material, such as litter, dead roots or root exudates into the soil is quite common in terrestrial ecosystems (Kramer et al., 2010; Yagi and Minami, 1990; Zhu and Cheng, 2011), and it is important for maintaining soil fertility (Sass et al., 1991; Schütz et al., 1989; Yagi and Minami, 1990). Moreover, input of fresh organic matter may accelerate or suppress soil organic matter (SOM) decomposition, causing a positive or negative priming effect (PE) (Guenet et al., 2010; Kuzyakov et al., 2000; Langley et al., 2009; Paterson et al., 2008; Wolf et al., 2007). A positive PE increases the rate of SOM decomposition (Chen et al., 2014; Paterson and Sim, 2013; Pausch et al., 2013; Zhu and Cheng, 2011). Negative PEs, which decrease the rate of SOM decomposition, are not reported quite as often as positive PEs (Cheng, 1996, 1999), but negative PEs are also of great significance to carbon balance, since slower decomposition leaves more C sequestered and not released as CO₂ (Kuzyakov et al., 2000). Over long time scales, PEs are

thought to be able to influence ecosystem C balance (Wieder et al., 2013). In addition, soil C pools are larger than the pool of atmospheric CO₂, so that small changes in the rate of soil C decomposition could cause a profound impact on atmospheric CO₂ concentration (Davidson and Janssens, 2006; Smith et al., 2008).

Underlying mechanisms of PEs remain largely elusive. Soil microorganisms, including bacteria and fungi, are considered to play the key role in the process leading to PEs during decomposition of upland SOM (Fontaine and Barot, 2005; Kuzyakov, 2010; Nottingham et al., 2009). It is widely accepted that the growth of microorganisms utilizing fresh organic matter (FOM degraders) is stimulated after substrate addition, followed by the gradual increase in the abundance of microorganisms utilizing polymerized SOM (SOM degraders), thus resulting in a positive PE (Fontaine and Barot, 2005; Fontaine et al., 2003; Perveen et al., 2014). In contrast, it is assumed that SOM degraders would preferentially utilize fresh organic matter, if it is available in excess, and thus lead to a negative PE, since competition between FOM and SOM

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degraders is negligible under this condition (Blagodatskaya et al., 2007; Cheng, 1999; Kuzyakov and Bol, 2006); however, experimental support remains ambiguous with some reports being inconsistent with these explanations (Rousk et al., 2015; Wild et al., 2014; Wu et al., 1993). Furthermore, recent studies suggest that there is a close correlation between PE and the soil microbial community composition. For example, diversity and composition of the soil microbial community were found to change in concert with negative or positive PE after single or repeated substrate amendments (Mau et al., 2015), and the magnitude of positive PE of fresh organic carbon on N mineralization from SOM increased in treatments with higher fungal dominance (Rousk et al., 2016). Despite this, it is still unclear what role the microbial community composition plays in causing a PE, in particular which microbial species are involved.

Current reports on PE and the plausible mechanisms were mostly targeted at various upland soils where CO₂ is the only end product of organic matter decomposition (Kuzyakov and Bol, 2006; Zhu and Cheng, 2011), but the PE has rarely been studied in flooded soil, such as rice field and wetland soils (Ye et al., 2015; Yuan et al., 2014), where both CO₂ and CH₄ are the end products of anaerobic degradation of organic matter. Anaerobic degradation is accomplished consecutively by a complex microbial community consisting of hydrolytic, fermentative, syntrophic, homoacetogenic bacteria and methanogenic archaea (Conrad, 1999; Glissmann et al., 2001). Anaerobic degradation of organic matter in rice fields is one of the most important sources of atmospheric CH₄ (Conrad, 2009), which has approximately 25 times the global warming potential of CO₂ (Forster et al., 2007). Rice provides the staple food for half the world population (Kalbitz et al., 2013). Input of plant material, such as rice straw (RS) is common in the management of rice field soils (Sass et al., 1991; Yagi and Minami, 1990; Yuan et al., 2012). Consequently, an effect of rice straw on rice field SOM degradation could influence the global budgets not only of CO₂, but also CH₄. While 80–90% of the RS is decomposed within the first year (Neue and Scharpenseel, 1987), the SOM in rice field soils is rather refractory, and was found to decrease only little (6–17%) within 120 days of anoxic incubation (Yao et al., 1999). The RS applied might be of significance for the decomposition rate of SOM in rice field soils exerting either a positive or negative PE. Previous studies indeed reported either negative (Conrad et al., 2012) or positive (Ye et al., 2015; Yuan et al., 2014) effects of RS on the production of CH₄ from SOM, but largely neglected the production of CO₂, which is an essential part of the PE on SOM in flooded soils.

In this study, we investigated the microbial mechanisms underlying the effect of RS on methanogenic SOM decomposition in rice field soils. We used two rice soil samples (Fuyang and Uruguay), which were selected based on the fact that they both had a relatively high CH₄ production potential (Fernandez Scavino et al., 2013; Yuan and Lu, 2009), and since RS additions resulted in different responses in SOM decomposition in the two soils. A ¹³C-labeling technique was applied to determine the PE of RS on SOM decomposition (Yuan et al., 2014). Our hypothesis has been that microbial community composition is the key for the PE, i.e. differences in PE between soils are dependent on their distinct soil microbial community compositions rather than on their distinct soil physicochemical properties. To test this hypothesis, we manipulated the soil microbial community through reciprocal inoculation with non-sterile and sterile samples of Fuyang and Uruguay soils. In this way we intended to create the same microbial community (e.g., from Fuyang) in a soil background with different soil physicochemical characteristics (Fuyang versus Uruguay). Then, we analyzed the bacterial and archaeal community composition and abundance in these soil samples. Correlation-based co-occurrence networks analysis was employed to produce microbial functional modules, aiming to reveal the differences in functional groups between soils with and without PE.

2. Material and methods

2.1. Soil samples

Soils were collected from China (Fuyang) and Uruguay. The China soil (Fuyang) is a clay loam (soil type: hydrargic anthrosol) collected in 2007 from a rice field (30.1 °N, 119.9 °E) at the China National Rice Research Institute in Hangzhou (Rui et al., 2009). The Uruguay soil is a clay soil (soil type: planosol) sampled in 2011 from a field (32.49 °S, 53.49 °W) 70 km from the Instituto Nacional de Investigación Agropecuaria (INIA) at the city Treinta-y-Tres, Uruguay (Fernandez Scavino et al., 2013). The fields in Uruguay had a history in rotation management. The typical rotation is four consecutive years of cattle pasture followed by two consecutive years of flooded rice fields. The soil sample used in this experiment was taken after four years of cattle pasture prior to flooding. Nevertheless, the Uruguay soil can still be considered as a paddy soil, since a previous study has concluded that a stable methanogenic microbial community established in the Uruguay soil once pastures had been turned into management by pasture-rice alternation (Fernandez Scavino et al., 2013). The sampling for each soil was done by taking soil cores (0–10 cm depth) from the ploughing layer at three locations in the field. Since we did not intend to assess site variability within the original field sites, a composite sample was prepared by mixing the samples by hand from all the three sites. These composite samples were termed Fuyang (F) or Uruguay (U), respectively. The soil samples were air-dried and stored at room temperature (Frenzel et al., 1999; Ma et al., 2010). The storage of dried soil at room temperature has no significant effect on soil methane production capacity (Mayer and Conrad, 1990). The dry soil lumps were broken using a mechanical grinder, and sieved through a 0.5-mm stainless steel sieve to homogenize sample (Chidthaisong et al., 1999; Roy and Conrad, 1999). Chemical characteristics of the soil samples are shown in Table S1. Part of each soil sample was sterilized by γ -irradiation (30 kGy; ⁶⁰Co) (McNamara et al., 2003; Philippot et al., 2013). The sterility of the γ -irradiated soil was checked by following CH₄ release upon flooding. No CH₄ production was detected during the whole experiment (62 days in total).

2.2. Preparation of the rice straw

Preparation of the ¹³C-labeled rice straw (RSI and RSII) has been described previously (Yuan et al., 2012). The RSI and RSII were prepared for calculating the relative contributions of RS and SOM to CH₄ and CO₂ as described below. The $\delta^{13}\text{C}$ values of RSI (596.1‰) and RSII (885.0‰) were obtained by mixing desired amount of ¹³C-labeled ($\delta^{13}\text{C} = 1859.9\text{‰}$) and unlabeled ($\delta^{13}\text{C} = -27.6\text{‰}$) RS. All the RS derived from rice plants grown in the greenhouse, ¹³C-labeled RS was prepared by labeling the rice plants with ¹³CO₂ (Yuan et al., 2012). These rice plants were harvested at the late vegetative stage, then RS was dried and ground to powder. In soil applied with RSI or RSII, the $\delta^{13}\text{C}$ values of the produced CH₄ and CO₂ were always lower than that of the RS mixture even when both gases were almost exclusively (90–100%) produced from the added RS. Therefore, the RS mixtures were sufficiently homogeneous to prevent preferential decomposition of ¹³C-labeled (and presumably labile) components of RS (Yuan et al., 2014). The C/N ratio of labeled RS was 20. The determination of the soil organic carbon content and the stable isotopic signatures of dried plant (RS) were carried out at the Institute for Soil Science and Forest Nutrition (IBW) at the University of Göttingen, Germany.

2.3. Soil incubation and analytical techniques

Waterlogged soil microcosms were prepared not only from original Fuyang and Uruguay soil samples, but also from combinations of original and sterilized soils as follows: first, 5% original Fuyang soil was inoculated into 95% sterilized Fuyang soil (5%F + sF) and sterilized

Uruguay soil (5%F + sU), respectively; also, 5% Uruguay soil was inoculated into 95% sterilized Uruguay soil (5%U + sU) and sterilized Fuyang soil (5%U + sF), respectively. Two further combinations were prepared as controls: 5% sterilized Uruguay soil was added into 95% original Fuyang soil (5%U + F), and 5% sterilized Fuyang soil was added into 95% Uruguay soil (5%F + U). For each soil microcosm, a total of 4 g dry weight soil was prepared and flooded with 6 ml anoxic water in 26-ml pressure tubes as described before (Yuan et al., 2014). Tubes were closed with butyl rubber stoppers, sealed with aluminum crimps, then flushed with N₂ and incubated statically at 25 °C in darkness. The CH₄ and CO₂ production were measured during the incubation. The Fe (III) reduction was determined by measurement of the production of Fe (II) during the incubation (Yao et al., 1999). The soils were preincubated under anoxic conditions, in order to revive the microbial community and establish relatively stable methanogenic conditions to mitigate handling effects (Teh and Silver, 2006). After 40 days of preincubation, RS treatments I and II were prepared by adding same amount (8 mg) of RSI or RSII powder into each tube. Immediately after RS addition, the tubes were sealed again and flushed with N₂, after shaking by vortexing, re-flushed with N₂ to remove the residual CH₄ and CO₂. Finally, the tubes were incubated statically at 25 °C for 22 days. All the treatments were prepared in triplicate.

2.4. CH₄ and CO₂ analyses

At regular time intervals (day 3, 5, 7, 10, 15, 20 and 22), gas samples from the headspace of the tubes were analyzed for CH₄ and CO₂ using a gas chromatograph (GC) equipped with flame ionization detector (FID). The CO₂ was measured after conversion to CH₄ using a methanizer (nickel catalyst at 350 °C) (Penning and Conrad, 2007). Total amounts of gases in the headspace of the tubes were calculated from the partial pressures using the volume of the gas space and the gas constant. The amounts of CH₄ dissolved in the liquid were less than 3% of the total and were neglected. The tubes were opened at the end of the incubation, and the liquid was analyzed for pH. Then the total amounts of CO₂ dissolved (aq) in the liquid were calculated from the solubility constant of CO₂ ($1 \times 10^{-1.47} \text{ mol L}^{-1} \text{ bar}^{-1}$), those of bicarbonate (HCO₃⁻) were calculated from the solubility constant of CO₂, the pH, and the dissociation constant ($10^{-6.35}$) of bicarbonate (Stumm and Morgan, 1981). The sum of gaseous, dissolved and bicarbonate CO₂ was defined as total inorganic carbon (TIC).

Stable isotopic analyses of CH₄ and CO₂ were performed as described in an earlier study (Penning and Conrad, 2007) using GC-combustion-isotope ratio mass spectrometer (GC-C-IRMS) (Finnigan, Bremen, Germany). The $\delta^{13}\text{C}$ values of dissolved CO₂ ($\alpha_{\text{CO}_2(\text{aq})} = 0.9990$) and HCO₃⁻ ($\alpha_{\text{HCO}_3^-} = 1.0075$) were calculated from the $\delta^{13}\text{C}$ of gaseous CO₂ and the corresponding fractionation factors α (Stumm and Morgan, 1981). The values of $\delta^{13}\text{C}_{\text{CO}_2(\text{g})}$, $\delta^{13}\text{C}_{\text{CO}_2(\text{aq})}$ and $\delta^{13}\text{C}_{\text{HCO}_3^-}$ were used to calculate $\delta^{13}\text{C}_{\text{TIC}}$ using the mole fractions of the different CO₂ species (Penning and Conrad, 2006).

2.5. Contribution of SOM and RS to CH₄ and CO₂ in soil slurries with RS application

The calculations were the same as described earlier (Yuan et al., 2014). In brief, since the only difference between the treatments RSI and RSII is the $\delta^{13}\text{C}$ of the RS applied, the fraction of CH₄ produced from RS (f_{RS}) was calculated by:

$$f_{\text{RS}} = (\delta^{13}\text{C}_{\text{CH}_4\text{I}} - \delta^{13}\text{C}_{\text{CH}_4\text{II}}) / (\delta^{13}\text{C}_{\text{RS-I}} - \delta^{13}\text{C}_{\text{RS-II}}) \quad (1)$$

of which the $\delta^{13}\text{C}$ values were determined experimentally. The $\delta^{13}\text{C}_{\text{CH}_4\text{I}}$ and $\delta^{13}\text{C}_{\text{CH}_4\text{II}}$ were the $\delta^{13}\text{C}$ values of the CH₄ produced in the RS treatment I and II, respectively; the $\delta^{13}\text{C}_{\text{RS-I}}$ and $\delta^{13}\text{C}_{\text{RS-II}}$ are $\delta^{13}\text{C}$ of the RS carbon in treatment I (596.1‰) and II (885.0‰), respectively.

Next, the fraction of CH₄ production from SOM (f_{SOM}) can be

calculated, since in the RS treatment

$$f_{\text{RS}} + f_{\text{SOM}} = 1 \quad (2)$$

Finally, the amount of CH₄ production from SOM ($p_{\text{SOM,CH}_4}$) and RS ($p_{\text{RS,CH}_4}$) were calculated from the total amount of CH₄ produced (p_{CH_4}) and the fractions of CH₄ production from SOM (f_{SOM}) and RS (f_{RS}), respectively:

$$p_{\text{SOM,CH}_4} = f_{\text{SOM}} p_{\text{CH}_4} \quad (3)$$

$$p_{\text{RS,CH}_4} = f_{\text{RS}} p_{\text{CH}_4} \quad (4)$$

Analogous equations are valid for the fractions and amounts of CO₂ produced from SOM and RS in the rice soil microcosms.

2.6. DNA extraction and quantification of microbial abundance

DNA from the soil samples collected after the preincubation and at the end of incubation was extracted according to the lysis protocol described in the FastDNA[®] Spin kit for soil (Qbiogene, Germany). The quantitative PCR of bacterial 16S rRNA was performed following a protocol described previously (Stubner, 2002); the quantitative PCR of archaeal methyl coenzyme M reductase (*mcrA*) gene, which is characteristic and unique for methanogenic archaea, was performed as described previously (Angel et al., 2011). The gene copy numbers detected are a proxy for the abundance of the respective microbes.

2.7. Pyrosequencing of bacterial and archaeal communities

For tagged pyrosequencing of bacterial 16S rRNA gene fragments we used primers F515 and R806 (Bates et al., 2011), and primers Arch344F and Arch915 for archaeal 16S rRNA gene fragments (Casamayor et al., 2002; Yu et al., 2008). The forward primer of each combination contained a unique 6-bp barcode (Hernandez et al., 2015). The purified PCR product of each sample was pooled in an equimolar concentration for pyrosequencing. The 454-pyrosequencing was carried out at the Max Planck Genome Centre in Cologne (Germany) using a Roche 454 Genome Sequencer GS FLX+.

All raw sequences obtained from pyrosequencing were first analyzed with Mothur (v. 1.27) software package (<http://www.mothur.org/>) (Schloss et al., 2009), and the OTU table was created using the UPARSE pipeline (http://www.drive5.com/usearch/manual/uparse_cmds.html) (Edgar, 2013). Within this pipeline, sequences were first sorted based on barcodes and removed from further analysis if they were shorter than 200 bp, contained ambiguous bases or homopolymers greater than 6 bp in length. Chimeras were removed using UCHIME (Edgar et al., 2011). Operational taxonomic units (OTUs) were defined from the accepted sequences with 97% sequence similarity. Taxonomic classification was carried out with the naïve Bayesian classifier in Mothur using the Silva 16S rRNA reference database. Rarefaction curves and diversity indices including microbial community richness (Chao1), diversity (Shannon index) and coverage were calculated in Mothur. The OTU table was subsampled to the minimum number of sequences obtained for a sample prior to downstream analysis. The 454 pyrosequencing reads (raw data) were deposited under the study number SRP058834 in the NCBI Sequence Read Archive (SRA).

2.8. Heatmap analysis

The heatmap representation of the relative abundance of bacterial OTUs among samples was built using R (<http://www.r-project.org/>), as described previously (Deng et al., 2014). The OTU abundance table was Hellinger transformed (decostand function within the R vegan package) to diminish the influence of zero values and to give low weights to rare species (Legendre and Gallagher, 2001). Principal components analysis (PCA) was performed using prcomp and the result indicated that for bacterial OTUs, PC1, PC2 and PC3 explained 40%, 18% and 13% of the

variance, respectively. To select the OTUs explaining most of the differences between samples, the 40 bacterial OTUs with highest loadings of PC1, 18 OTUs of PC2 and 13 OTUs of PC3 were chosen to construct the heatmap. A total of 27 unique OTUs were obtained because of the two reasons: first, some of the OTUs were selected from more than one PC; second, OTUs with low averaged relative abundance ($< 1\%$ in every control or treatment) were removed (those removed OTUs also had relatively lower loading values). The OTU abundances were converted to percentage of reads from each sample and the heatmap constructed using the heatmap.2 function in gplots (Warnes et al., 2014). The taxonomy of the selected OTUs was added separately.

2.9. Co-occurrence network analysis

The bacterial and archaeal OTU tables were randomly subsampled to the same sequence depth using daisychooper.pl (<http://www.festinalente.me/bioinf/downloads/daisychooper.pl>). These tables were combined so as to prepare the network analysis for prokaryotic OTUs in soil samples. Cosmopolitan bacterial and archaeal OTUs, which occurred in more than half of the selected samples were used for network analysis. We calculated all possible Spearman's rank correlations between selected OTUs (Ju et al., 2014). We considered a valid co-occurrence event to be a robust correlation if the Spearman's correlation coefficient (ρ) was > 0.6 and the P value was < 0.01 (Barberan et al., 2012). Correlation networks were constructed with the robust correlations as weighted edges and visualized with Gephi software (<https://gephi.github.io/>). 10,000 Erdős-Rényi random networks, which had the same number of nodes and edges as the empirical networks, were generated using the R package igraph (<http://cran.rproject.org/web/packages/igraph/>) (Ju et al., 2014).

2.10. Statistical analysis

To test the significance of the differences between control and RS treatment on various variables, two-tailed independent t-tests were applied using Microsoft Excel 2007. The significance of differences in relative abundance of OTU between treatments was determined by one-way analysis of variance (ANOVA) using SPSS 13.0. P values below 0.05 were considered statistically significant.

Overall structural changes of prokaryotic communities were evaluated by Principal Coordinate Analyses (PCoA) with Fast UniFrac distances (Lozupone et al., 2006). The statistical significance among datasets was assessed by PerMANOVA using the weighted PCoA scores in PAST (<http://folk.uio.no/ohammer/past/>). The Mantel test was applied to evaluate the correlations between prokaryotic communities with environmental variables using the Mantel procedure in the R package Vegan. The Variance partitioning analysis (VPA) was performed to quantify the relative contributions of environmental variables to changes in the bacterial and archaeal community structures by the method described previously (Yao et al., 2014).

3. Results

3.1. Differential response of methanogenic decomposition of SOM to RS addition

The paddy soils were preincubated for 40 days under anoxic conditions to ensure that soil conditions were reduced and methanogenesis was the exclusive terminal decomposition process of organic matter, which was confirmed by active CH_4 production (Fig. S1) and absence of Fe (III) reduction (data not shown). Subsequently, the soils were amended with 0.2% ($2 \text{ mg straw g dw}^{-1} \text{ soil}$) ^{13}C -labeled RS, and the amount of CH_4 and TIC produced from SOM in the treatment were calculated using eqs. (1)–(3). The results showed that RS treatment enhanced the accumulation of SOM-derived CH_4 in Fuyang (F) soil sample (Fig. 1a). Nevertheless, there was no significant difference in the

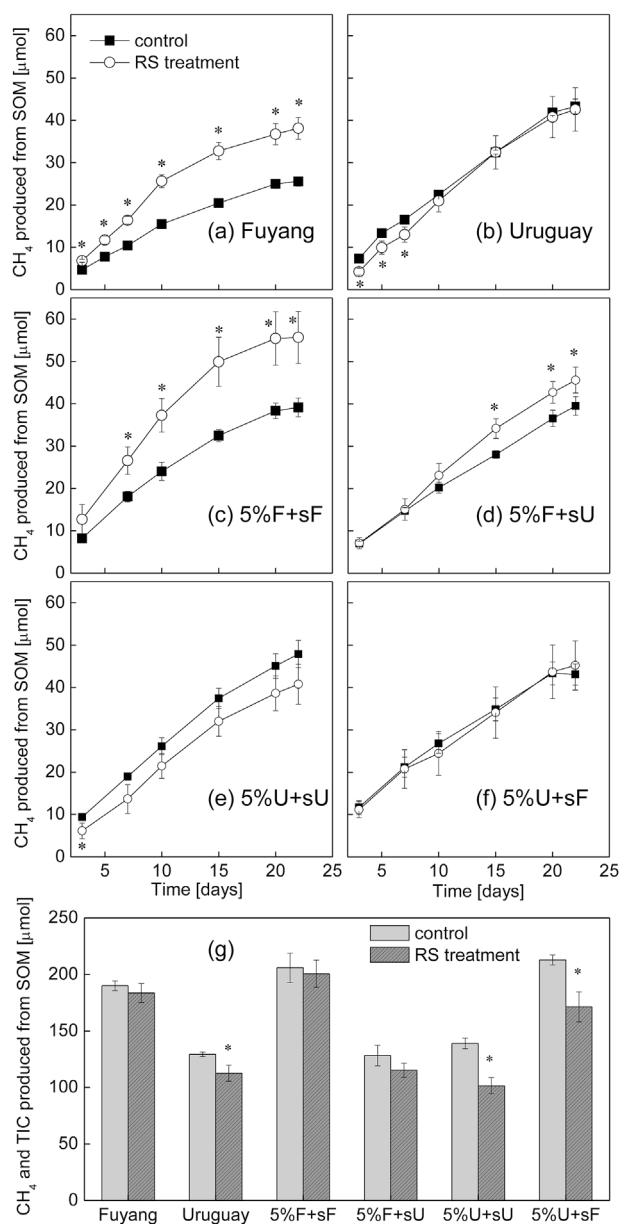


Fig. 1. The SOM-derived CH_4 and total inorganic carbon (TIC) production in control and RS amended soils. Development of SOM-derived CH_4 production from Fuyang (a), Uruguay soil (b), from the combinations of 5%F with sterilized F (5%F + sF) (c) or with sterilized U (5%F + sU) (d), and from the combinations of 5%U with sterilized U (5% U + sU) (e) or with sterilized F (5%U + sF) (f) in control and rice straw (RS) treatment. Total amount of SOM-derived CH_4 and TIC at the end of incubation (day 22) from Fuyang, Uruguay, and from the combinations of original with sterilized soils in control and RS treatment (g). The data shown are from treatment RSI, which however was almost identical to the results from treatment RSII. Data are means \pm SD ($n = 3$). The differences between control and RS treatments were tested by two-tailed independent t-tests, indicated by * when $P < 0.05$.

total amounts of SOM decomposition (SOM-derived CH_4 and CO_2) between RS treatment and control at the end of incubation (Fig. 1g), since the accumulation of CO_2 (quantified as TIC) was decreased (Fig. S2). In Uruguay (U) soil sample, however, the SOM-derived CH_4 was almost the same between treatment and control (Fig. 1b), while SOM decomposition was significantly decreased in the RS treatment (Fig. 1g). Besides, the total amount of SOM decomposition in F soil ($> 183 \mu\text{mol}$) was substantially larger than that in U soil ($< 130 \mu\text{mol}$), which is consistent with the higher soil organic carbon content in F (2.38%) than U soil (1.57%) (Table S1).

In both combinations of 5% non-sterilized F with sterilized soil (sF

or sU), the effects of RS addition on both SOM-derived CH₄ production and SOM decomposition (Fig. 1c, d, g) were similar to that in original F soil, although the amount of SOM decomposition in combination of 5% F with sU was similar with that in original U soil. For both combinations of 5% U with sterilized soil (sF or sU), the effects of RS were consistent with that in original U soil (Fig. 1e, f, g), although the amount of SOM decomposition in combination of 5%U with sF was similar with that in original F soil. On the other hand, for the combination of 5% sterilized U with original F, the effects of RS were consistent with that in original F soil (Figs. S3a and c), and vice versa (Figs. S3b and c). The experiment of sterilization and inoculation had little influence on the pH values of soil samples (Table S2). Besides, the amounts of RS decomposition were not significantly different between original F soil and U soil, and between sterilized soil inoculated with F and U (Fig. S4). However, CH₄ production was enhanced in sF inoculated with 5%F compared to the original F soil (Fig. 1a, c), while SOM degradation was similar (Fig. 1g). Sterilization of the F soil apparently resulted in enhanced production of CH₄. This was probably caused by a side effect of gamma irradiation. It has been shown that decomposition rates in some γ -irradiated soils, after re-introduction of a microbial community, were greater than in the un-irradiated controls (McNamara et al., 2003).

3.2. Abundance of bacterial and archaeal communities

The abundances of bacteria and methanogenic archaea in most of the soil samples were in the similar range after preincubation before being used for the incubation experiments (Fig. S5). This was also the case for the sterilized soils that had been inoculated with 5% non-sterile soil, indicating that the microorganisms had increased in abundance during the preincubation time. The microbial abundances were again determined at the end of the incubation experiment using quantitative PCR. The abundance of the bacterial 16S rRNA gene ranged from about 3×10^9 to 9×10^9 copies g⁻¹ soil (Fig. 2a). RS addition significantly stimulated the abundance of bacteria only in original F and in the combination of 5%U with sterilized F. The abundance of methanogenic archaea was determined by targeting the methyl coenzyme M reductase

(*mcrA*) gene, which is characteristic and unique for methanogenic archaea. The results indicated that original U soil had the lowest abundance of *mcrA* (5.6×10^7 copies g⁻¹ soil) (Fig. 2b), while the highest abundances of *mcrA* were found in 5%F + sF with RS treatment (3.3×10^8 copies g⁻¹ soil). The RS treatment resulted in significant increase of the *mcrA* abundance in the original U soil only.

3.3. Bacterial and archaeal 16S rRNA genes diversity analysis

A similarity level of 97% was used to identify OTUs and to estimate diversity of bacterial and archaeal 16S rRNA genes in soil samples. A total of 122,201 bacterial sequences corresponding to 7987 OTUs were obtained after quality filtering. In both control and RS treatment of F soil and control of U soil, about 2000 OTUs were found at a sequencing depth of about 7000 (Fig. S6a), while all other soil samples contained fewer than 900 OTUs at a sequencing depth ranging from 1272 to 2611. Rarefaction curves of Shannon indices almost approached plateaus (Fig. S6c) and the coverage was higher than 80% in each soil sample (Fig. S6d), although the rarefaction curves of OTUs and Chao1 were not saturated in any sample (Figs. S6a and b). The control and RS treatment of original F soil and the control of original U soil were sequenced to greater depth, since they displayed a higher bacterial α -diversity than the other samples. The dominant phyla were Firmicutes (39.9% on average) and Proteobacteria (12.6% on average) (Fig. 3a). The major difference between the original F and U soil was the relative abundance of Firmicutes being higher in F soil. The sterilization and inoculation treatments increased the relative abundance of Firmicutes in each soil. RS treatment increased the relative abundance of Acidobacteria and Firmicutes in original U soil, while there was no major difference between control and RS treatment in other soil samples.

For the archaea, a total of 106,220 sequences belonging to 424 OTUs were obtained after quality filtering. The sequencing depths ranged from 1143 to 6123, and the sequences from different samples clustered into 73 to 173 OTUs. The sequence sampling effort was sufficient to obtain coverages higher than 97% for every sample (data not shown). The archaeal communities were composed of both Crenarchaeota and Euryarchaeota phyla (Fig. 3b). Euryarchaeota-affiliated sequences were dominant in most of the samples, accounting for more than 90% of archaeal sequences except in the original Uruguay soil (82%).

3.4. OTU-level bacterial and archaeal diversity analysis

The bacterial communities in sterilized soils were highly correlated (79%) with the inoculum sources (Table 1). Variance partitioning analysis (VPA) also showed that inoculum source explained a higher ratio of the observed variation (37%) of bacterial communities relative to other variables including soil type, etc (Table S3). Indeed, soil type affected the soil bacterial community composition only little (17%) albeit significantly (Table S3). As a result, bacterial communities of the soil samples were separated into two clusters (F and U) in accordance with the inoculum sources based on Unifrac distances (PerMANOVA $p = 0.001$) (Fig. 4). One replicate of 5%U + sF apparently deviated from the others and was close to cluster F. We assumed that this variation was caused by the treatment of sterilization and inoculation. In contrast to the Bacteria, the archaeal communities did not cluster according to the origin of the microbial communities (Fig. S7), and the inoculum source also explained less of the variance (26%) of the archaeal communities (Table S3). Next, the heatmap analysis was used to intuitively display the differences in relative abundances of bacterial OTUs among samples (Fig. 5). The OTUs with the highest contribution to the PCA ordination were selected. The results indicated that the relative abundance of OTU2 and OTU52 (both belonging to *Symbiobacterium*) significantly increased in original U soil after RS addition (Table S4); moreover, both these OTUs were also much higher in sterilized soils inoculated with U than with F soil (Table S5). In

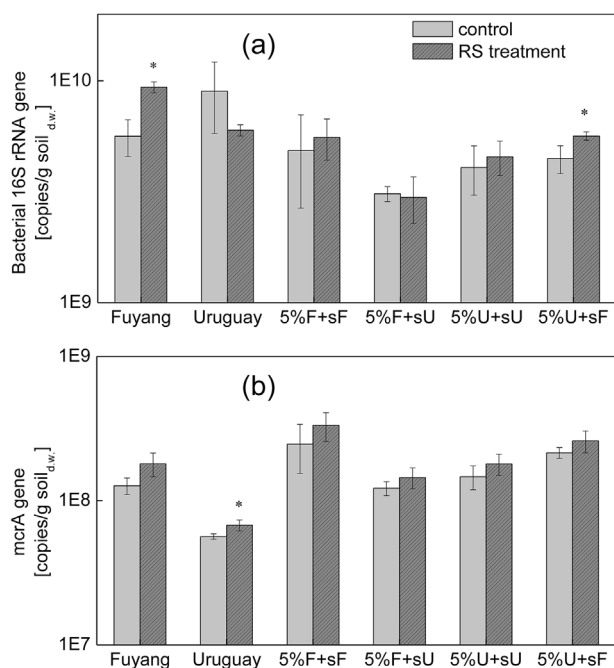


Fig. 2. Bacterial 16S rRNA gene (a) and *mcrA* gene (characteristic for methanogenic archaea) (b) copy numbers in soils without and with RS application at the end of incubation (day 22); means \pm SD ($n = 3$). The difference between control and RS treatments of each soil sample was examined by two-tailed independent t-tests, indicated by * when $P < 0.05$.

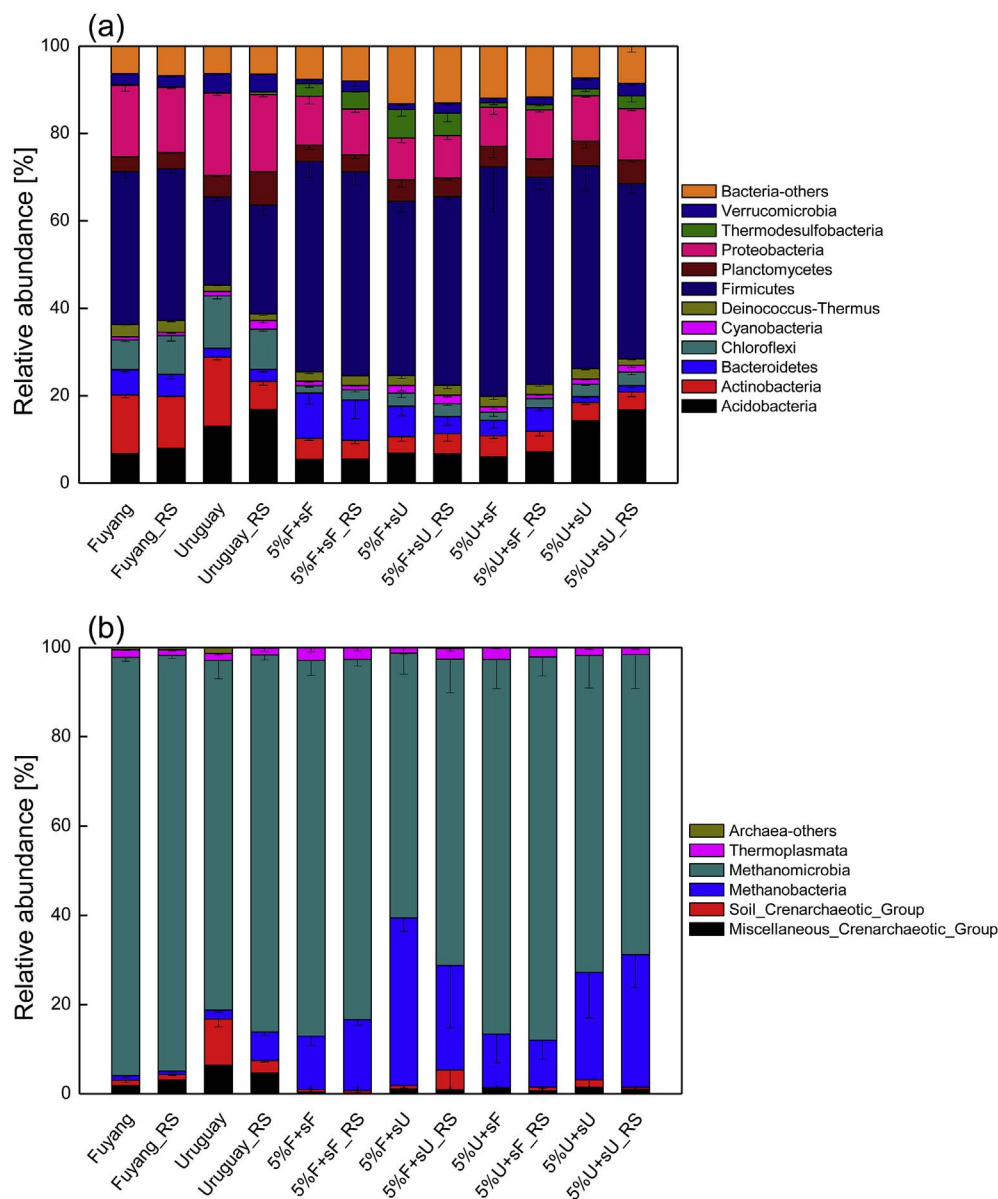


Fig. 3. Relative sequence abundances of bacterial phyla (a) and archaeal classes (b). Major taxa detected with average relative sequence abundances > 1% are displayed; means ± SD (n = 3). Column “Bacteria-others” or “Archaea-others” indicate combined relative sequence abundances of all the rare phyla or classes, candidate divisions and of the taxonomically unclassified sequences; rare phyla or classes are defined as having average relative sequence abundances between all samples of < 1%. The description of soil samples was the same as in caption of Fig. 1. There were control and RS treatment for each soil sample.

contrast, the relative abundances of OTU8026 (*Clostridium*), OTU1 and OTU3 (both belonging to Sphingobacteriales) were significantly higher in original F compared with original U soil. OTU3 in particular was notably more abundant in sterilized soils inoculated with F than with U soil (Table S5).

3.5. Network analysis of cosmopolitan bacterial and archaeal OTUs in cluster F and U

Two positive correlation-based networks, named F and U (Fig. 6a and b), were constructed with these cosmopolitan prokaryotic OTUs of

samples in cluster F and U (Fig. 4), respectively. The results showed that network F and U were similar in size and topology (Table S6), sharing about 40% of the bacterial nodes and about 90% of the archaeal nodes (Fig. S8). In each network, there were three major modules (Fig. 6 and Table 2). Among them, two major modules were mainly composed of bacterial nodes, i.e. FM1 and FM2 in network F, UM1 and UM2 in network U (Table 2), while another module in each network was almost exclusively composed of methanogenic archaea (FM3 and UM3). In network F, bacterial nodes in FM2 had numerous positive correlations with methanogens in FM3 (Fig. 6), the same as between UM2 and UM3 in network U. The modules FM1, FM2, UM1 and UM2 exhibited

Table 1
Spearman's correlation of environmental variables with bacterial and archaeal communities of all soil samples treated with sterilization and inoculation. Mantel test was applied in this analysis. The relative abundances of OTUs were used as input. RS: rice straw.

	Bacterial community				Archaeal community			
	Soil type	Inoculum source	RS treatment	Averaged CH ₄ production rate	Soil type	Inoculum source	RS treatment	Averaged CH ₄ production rate
Correlation	0.33	0.786	-0.001	0.055	0.118	0.384	-0.025	0.014
p value	0.003	0.001	0.352	0.146	0.033	0.001	0.617	0.363

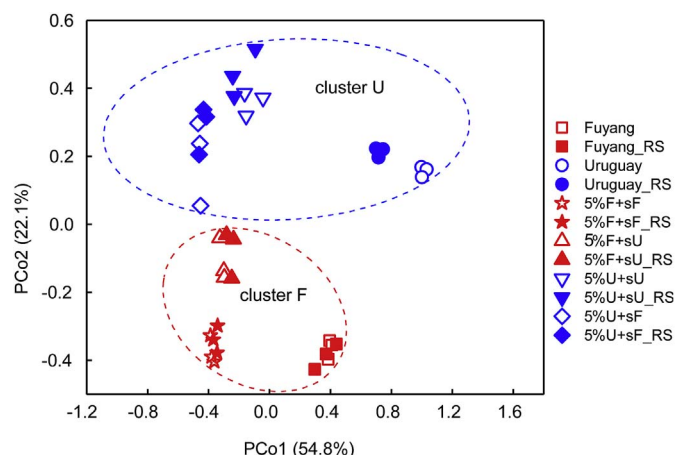


Fig. 4. Principal coordinate analysis (PCoA) of bacterial communities based on weighted UniFrac metrics. The description of soil samples was the same as in caption of Fig. 1. There were control and RS treatment for each soil sample.

positive correlation with soil organic carbon (SOC) content and soil pH value but not with RS treatment (Table 3).

Symbiobacterium was dominant in network U but substantially lower in network F (32% versus 3%) (Table S7). Besides, in network F, the number of positive correlations between bacteria and hydrogenotrophic methanogens was similar with that between bacteria and acetoclastic methanogens (Table S8). In network U, however, both these correlations were more numerous, and in addition the number of positive correlations between bacteria and acetoclastic methanogens exceeded that between bacteria and hydrogenotrophic methanogens.

4. Discussion

Addition of fresh organic matter in form of RS to anoxic flooded soils affected SOM degradation to CO₂ plus CH₄. In F soil, RS input had

no effect on SOM decomposition (no PE; Fig. 1g). However, the relative amount of CH₄ produced from SOM significantly increased (Fig. 1a). Consequently, the production of CO₂ from SOM must have decreased. This observation is best explained by assuming that hydrogenotrophic methanogenesis from SOM-derived CO₂ was stimulated by H₂ released from RS decomposition, similarly as it had been observed in Italian rice field soil (Yuan et al., 2014). By contrast, stimulation of acetoclastic methanogenesis should have increased production of both CO₂ and CH₄. In U soil, however, RS addition resulted in suppression of SOM degradation (negative PE) to CO₂ and CH₄ (Fig. 1g) and thus, decreased greenhouse gas production from SOM.

Reciprocal inoculation of sterilized soil with non-sterile soil from the same or the different type showed that the PE on SOM degradation was predominantly determined by the soil inoculum source. For example, both sterilized F and U soils showed negative PEs after inoculation with 5% U but not with 5% F (Fig. 1g). Therefore, the observed PE was not caused by the physicochemical soil characteristics but by the soil microbial community. This conclusion is at least true for the soils studied. However, we cannot exclude that in other rice cultivation areas soil physicochemical characteristics may be of greater importance for causing PE.

RS treatment significantly increased the abundance of bacteria or methanogens only in some of the soil samples, which did not necessarily have a PE (Fig. 2a and b). Hence, in our soils it is unlikely that the observed PE was caused by the biomass of bacteria or methanogenic archaea. This finding does not rule out, however, that in other soils acceleration or retardation of SOM decomposition may be due to the increase of soil microbial biomass after substrate addition (Kuzyakov et al., 2000).

We conclude that in our experiments it was the composition of the soil microbial communities rather than biomass abundance or physicochemical soil characteristics that did or did not cause PE (Fig. 1, 2 and 4). In order to characterize the correlation of bacterial and archaeal microorganisms in methanogenic soils with and without a PE, two positive correlation-based networks were constructed with

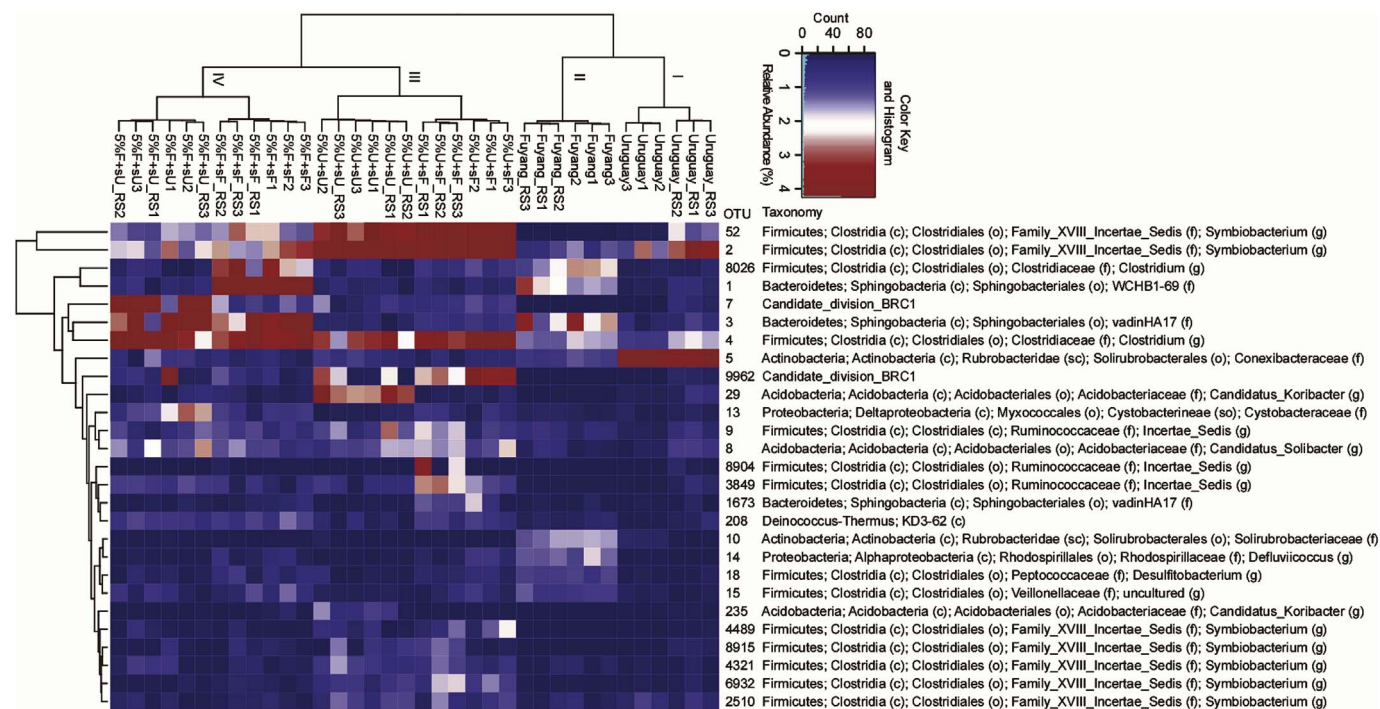


Fig. 5. Heatmap showing the relative abundance of selected bacterial OTUs. The colors correspond to the relative abundance of the OTUs in the samples, as indicated by the color legend. The samples are clustered according to Bray–Curtis distances. The taxonomy of each OTU is provided to the lowest level obtained during the classification. Abbreviations are used to indicate class (c), subclass (sc), order (o), subclass (so), family (f) and genus (g). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

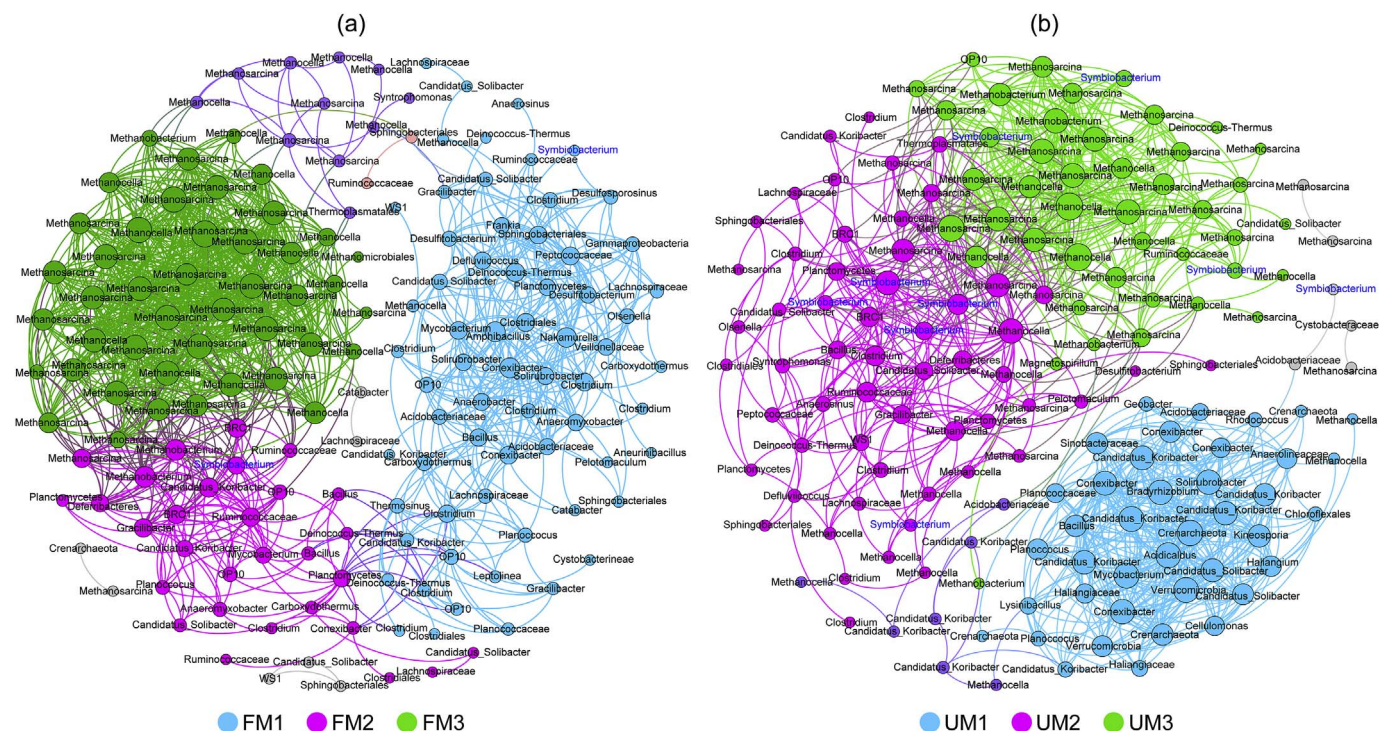


Fig. 6. Networks of co-occurring prokaryotic OTUs in soil samples based on correlation analysis. The network F (a) includes both the samples derived from original F soil and the sterilized soils with 5% F, the network U (b) includes both the samples derived from original U soil and the sterilized soils with 5% U. Nodes were colored by modularity class with labeled taxonomic affiliation. The names of three major modules in each network are shown, while those of other minor modules are neglected. A connection between two nodes (edge) stands for a strong (Spearman's $\rho > 0.6$) and significant ($p < 0.01$) correlation. For each panel, the size of each node is proportional to the number of connections (degree). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2

Taxonomic information of dominant modules in the networks of F and U, the numbers of nodes affiliated with abundant class/order were shown in the table, the numbers in parentheses were the total numbers of nodes in each module. FM: major module in network F, UM: major module in network U.

	Network F			Network U		
Abundant Class/Order	FM1 (66)	FM2 (30)	FM3 (44)	UM1 (40)	UM2 (57)	UM3 (44)
Clostridia	28	9			21	4
Acidobacteria	7	4		9	3	1
Actinobacteria	8	2		9	1	
Bacilli	5	3		5	1	
Sphingobacteriales	2				3	
Methanocellales	2		12	2	9	8
Methanosarcinales		1	30		8	24
Methanobacteriales		2	1			4

Table 3

Spearman's correlation of environmental variables to prokaryotic community structures of dominant modules in networks tested by Mantel test (permutations: 9999). RS: rice straw, SOC: soil organic carbon.

	pH	RS treatment	SOC content
(1) network F			
FM1	0.342**	-0.053	0.457**
FM2	0.482**	-0.035	0.694**
FM3	-0.03	-0.055	-0.016
(2) network U			
UM1	0.426**	-0.037	0.520**
UM2	0.405**	0.067	0.470**
UM3	-0.086	0.052	-0.099

* $p < 0.05$, ** $p < 0.01$.

cosmopolitan prokaryotic OTUs for cluster F and U, respectively (Fig. 6a and b). Correlation-based co-occurrence network analysis can produce microbial functional modules, which allows the interactions between different functional groups in complex systems to be revealed

(Barberan et al., 2012; Deng et al., 2012; Ju et al., 2014). The prokaryotic community in each network was organized by three major functional modules (Fig. 6 and Table 2). Values of modularity, average clustering coefficient and two other parameters in both empirical networks were higher than those in random networks (Table S6), suggesting that the empirical networks had common network characteristics, such as modularity and hierarchy properties (Deng et al., 2012; Watts and Strogatz, 1998).

The important function of each module could be inferred based on the prokaryotic composition and their known physiological functions (Rui et al., 2015). Our results suggested that each network probably included a primary fermentation module (FM1 or UM1), methanogenic fermentation module (FM2 or UM2) and methanogenic module (FM3 or UM3). This was consistent with studies showing that anaerobic methanogenic systems consist of well-organized, closely interacting bacterial and archaeal populations (Kim and Liesack, 2015; Rui et al., 2015). Most of the nodes in FM1 and UM1 belonged to Firmicutes, Actinobacteria and Acidobacteria (Table 2), which usually are involved in hydrolysis of complex organic matter (Kim and Liesack, 2015; Rui

et al., 2015; Wegner and Liesack, 2016). The numerous positive correlations between bacteria and methanogens in FM2 and UM2 were probably caused by the bacterial production of methanogenic substrates, e.g., H_2/CO_2 , formate and acetate (Kim and Liesack, 2015; Rui et al., 2015), or because of multiple syntrophic interactions between bacteria and hydrogenotrophic methanogens (Schink, 1997). In general, positive co-occurrence of prokaryotic populations within or between modules could reflect their similar niche adaptation or inter-species cooperation (Rui et al., 2015).

The network analysis mainly reflected the correlation of prokaryotic populations during degradation of SOM rather than degradation of RS because of two reasons. First, RS addition did not substantially change the bacterial and archaeal community compositions and abundances in most of the soil samples (Figs. 2, 3 and S4). Second, the two types of fermentation modules in both networks were highly correlated with the SOC content but not with the RS treatment in each soil (Table 3). Therefore, we suggest that the network analysis is helpful in the elucidation of differences in the microbial community composition between soils with and without PE during SOM decomposition.

For example, the primary fermentation module (FM1) of network F contained more than 20 nodes of Clostridia (Table 2). However, in network U, OTUs of Clostridia were solely found in the methanogenic fermentation module (UM2). Clostridia are of importance for the anaerobic breakdown of polymers in flooded paddy soil, but could also participate in fermentation and utilization of sugar (Kim and Liesack, 2015; Rui et al., 2015). Therefore, some major nodes of the Class Clostridia, such as the genus *Clostridium* (Fig. 6 and Table S7), probably were involved in the primary fermentation and methanogenic fermentation during consecutive degradation of SOM in soils of F and U clusters, respectively.

Analogously, *Symbiobacterium* was dominant in network U but substantially lower in network F (Table S7), which was also consistent with the heatmap analysis of the bacterial OTUs in soils of F and U clusters (Fig. 5). *Symbiobacterium* spp. are known as symbiotic bacteria (Ohno et al., 2000; Rhee et al., 2002), but also exhibit marked monogrowth if CO_2 or bicarbonate is available (Watsuji et al., 2006). *S. thermophilum* possesses a glucose degradation pathway and carries the genes for metabolizing gluconate, cellobiose and others (Ueda et al., 2004). Indeed, nodes of *Symbiobacterium* in network U had positive correlations with numerous bacteria and methanogenic archaea (Fig. 6). *Symbiobacterium* was actively involved in methanogenic fermentation in soils of U cluster, in accordance with its presence in modules UM2 and UM3, while this was not the case in soils of F cluster. Finally, compared with network F, bacteria in network U had more positive correlations with methanogens, especially having many more edges with acetoclastic than with hydrogenotrophic methanogens (Table S8). These results implied that the two networks probably differed in the role of hydrogenotrophic methanogenesis versus acetoclastic methanogenesis, which suggested that these soils of F and U clusters had different pathways of methanogenic degradation of SOM (Conrad et al., 2009, 2010). Therefore, network analysis of prokaryotic community composition indicated that bacterial communities involved in consecutive anaerobic SOM decomposition were apparently different between soils of F and U clusters. The reason why such difference in microbial community composition and network clustering exists between F and U soils is unknown. In fact, it is generally not known how and why differences in the individual microbial communities arise between different soils. Generation of such knowledge needs much more research using a large variety of different soils.

The mechanism for the negative PE in soils of U cluster is not quite clear, but our results allow some speculation. It has been proposed that a negative PE may be due to a switch of the SOM-degrading microorganisms from degradation of SOM to degradation of the fresh organic matter added (Blagodatskaya et al., 2007). We speculate that *Symbiobacterium* and other Clostridia, which were prevalent in soils of U cluster and were probably involved in methanogenic fermentation,

were able to switch to FOM degradation, while the bacteria that were characteristic for soils of F cluster were not able to do so. This interpretation is consistent with the observation that the bacteria in network U had more positive correlations with both hydrogenotrophic and acetoclastic methanogens than in network F (Table S8), so that a switch from SOM to FOM would have immediate effects on production of both CH_4 and CO_2 , now being produced from FOM instead SOM. This was consistent with the negative PE in soils of U cluster after RS addition. In soils of F cluster, by contrast, FOM degradation would only indirectly affect the methanogens in network F, resulting in stimulation of CH_4 production but not of SOM degradation. Besides, it's not likely that the accumulated intermediates inhibited decomposition of both RS and SOM in the soils of U cluster, since the amount of decomposition of RS in soils of U cluster were almost the same with that in soils of F cluster (Fig. S4).

In summary, the bacterial community composition (and its activity) was found to be crucial for establishment of a negative PE on SOM degradation in Uruguay rice field soil. This result is consistent with our hypothesis that the PE in a soil mainly depends on its soil microbial community rather than its physicochemical properties. It is unclear, however, whether this result is universal for rice field soils. Production of the greenhouse gas CH_4 is always considerably higher in the presence than in the absence of RS (Conrad and Klose, 2006; Kimura et al., 2004; Peng et al., 2008). Nevertheless, large amounts of SOM would be preserved and the emission of greenhouse gases (CH_4 and CO_2) reduced if the bacterial community composition would be optimal for avoiding a stimulating and causing a suppressing effect. Our results showed that tight networks between fermenting and methanogenic microorganisms may facilitate a switch between the degradation of SOM to degradation of fresh organic matter (e.g., RS) thus preserving SOM. Such tight methanogenic networks seem to depend on the presence or absence of particular bacterial genera, e.g. *Symbiobacterium*. On the other hand, previous studies in upland soils have shown that several factors, including quality and quantity of SOM, do also matter for positive and negative PEs (Kuz'yakov et al., 2000). Similarly, there must remain many unknowns regarding the PEs in rice soils that cannot be understood from the two soils investigated for this study. Therefore, further studies on more rice field soils are necessary for fully uncovering the mechanisms of PE during methanogenic decomposition of SOM.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.soilbio.2017.10.004>.

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