Changes in 16S rRNA bacterial community structures after C and N additions – comparison of organic farmed and conventionally farmed soils

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Abstract
The use of legumes has received heightened interest as an alternative to chemical fertilizers in recent years. It adds not only nitrogen (N) but also carbon (C) to soils thus it might influence the soil microbial community structures. Considering that microorganisms play a key role in N dynamics, it is important to understand the relationship between the soil microbial structure and soil N dynamics, in relation to the use of legumes. Thus, we aimed to evaluate the relationship between the long-term legume application and soil microbial community structures. Also we aimed to investigate the relationship between the microbial communities and the soils' N immobilization potential, because N immobilization by microbes often competes with plants in terms of N availability in soils. We compared a soil with the history of the use of green manure (hairy vetch, HV) and a soil with the history of the use of N chemical fertilizer (CF). First, we investigated the microbial communities using a colony counting method and a 16S rRNA gene analysis. Then we conducted an incubation experiment where we added C and N source into soils and measured N immobilization potentials. Bacterial community structures were analyzed to investigate the interaction between N immobilization potentials and bacterial community structures. Our results showed that there was a significant difference in microbial community structures for the two soils before the addition of C and N. However, no significant difference was detected on N immobilization potential during the incubation. Moreover, the difference between the bacterial community structures in the two soils became smaller as the incubation progressed, within 14 days. According to these results, it is indicated that microbial community structures were clearly influenced by the use of hairy vetch but with added C and N, the community structure differences due to the use of hairy vetch might disappear.

Key Words
16S rRNA , bacterial community structure, nitrate

Introduction
Nitrogen (N) is one of the most important nutrients for plant growth, thus to maintain agricultural productivity, chemical fertilizer is commonly used as N source. Alternatively, green manure legumes have been receiving attention as N source. Nitrogen is also an important nutrient for soil microbes to maintain their activity. Microbes can also be sinks for N and the N within live-microbes (biomass N) cannot be utilized by plants. The incorporation of plant available N into microbes is called N immobilization and high N immobilization activity may result in the loss of agricultural productivity. Thus, the use of green manure legumes may lead to increased available N in soils but it is also important to understand the potential influence on soil microbial N immobilization potential. However, there are few studies performed to understand the changes in soil microbial N immobilization rates and amount due to the use of green manure legumes. Consequently, the objects of this study were (1) to evaluate the relationship between long-term legume application and microbial community structure and (2) to obtain information about the relationship between microbial community and microbial N immobilization in terms of legume application.

Methods
Soil sampling sites
Soil (5 cm depth, Alluvium (brown lowland soil)) was collected from a plot trial in a greenhouse at an experimental greenhouse, at the Field Science Center for Northern Biosphere, Hokkaido University, Sapporo, Japan (43°3'N, 141°20'E), in November 2014, after the harvest of the tomato crop. The plot trial consists of five treatments (chemical fertilizer, hairy vetch [*Vicia villosa*] mulch, oats [*Avena strigosa*] mulch, hairy vetch plus oats mulch, and hairy vetch incorporated), with three replicates. The plot trial had been established in 2006 and lasted for 9 years. In the current experiment, we sampled soils from only "chemical fertilizer (CF)" and "hairy vetch mulch (HV)" plots (two treatments × three replicates).

Experiment 1. Microbial analyses of soils

The amount of bacteria and fungi in the sampled soils were estimated by colony count methods. To investigate the microbial community, 16S rRNA based analysis was conducted using the Ion PGM systems (Life Technologies, Inc.) following manufactures instructions. Briefly, the extracted soil DNA was amplified targeting the V2–4–8 and V3–6, 7–9 regions of the 16S rRNA gene, using the Ion 16S Metanogenomics kit and final template was sequenced with the Ion 316 / 314 chip.

Experiment 2. Laboratory experimental setup for the measurement of N immobilization
Using the two soils sampled from the tomato greenhouse, an incubation experiment was performed to investigate the differences in N immobilization potentials when excess C was applied. The experimental set up was 2 soil types (CF and HV) × ± N application (1 g N kg^{-1} soil as KNO_3-N) × ± straw application (100 g rice straw kg^{-1} soil) with three replicates. Every 2 days, following the start of the incubation, the decrease in nitrate (NO_3^--N) concentrations and the increase in soil microbial biomass carbon (MBC) were measured as N immobilization potential indicators. Nitrate concentrations were measured using a colorimetric method with a flow injection analyser (AQLA-700, Aqualab Co., Ltd., Japan) and MBC was measured with chloroform fumigation method. For the subsamples taken at day 1, 5 and 14 during the incubation experiment (soils with added C and N), the soil bacterial community structures were determined using the 16S rRNA based method, as described above.

Results
Experiment 1. Microbial analyses of the sampled soils
The total counts of bacteria was, on average, over 2.0-fold higher in the HV soil ($p< 0.05$), on the other hand, for fungi, the number of colonies was, on average, 2.0-fold higher in the CF soil ($p< 0.05$) (data not shown). Based on the 16S rRNA analyses, there were six dominant phyla and *Proteobacteria*, *Actinobacteria* and *Gemmatimonadetes* were dominant in HV soil, whereas *Cyanobacteria* was dominant in CF soil ($p< 0.05$). Based on the similarly analyses, the two community structures were significantly different (Fig. 1).

Experiment 2. Incubation experiment
With added C and N, the amount of NO_3^--N rapidly decreased and the decreasing rates were not different between the two soils (data not shown). Also, MBC did not show different responses between the two soils when C and N were applied, although the MBC was HV > CF without C and N additions (data not shown). However, with added C and N, the microbial community structures markedly changed. Dominated phylum changed its composition from that of original soil. On day 1, the ratio of *Preteobacteria*, *Firmicutes* and *Bacteroidetes* were significantly different between soil types ($p < 0.05$) (Fig. 2). On day 5, the ratio of *Bacteroidetes* and *Proteobacteria* were significantly different between soil types ($p < 0.05$) (Fig.2). On day 14, significant difference was found only with *Actinobacteria* ($p < 0.05$) (Fig. 2).

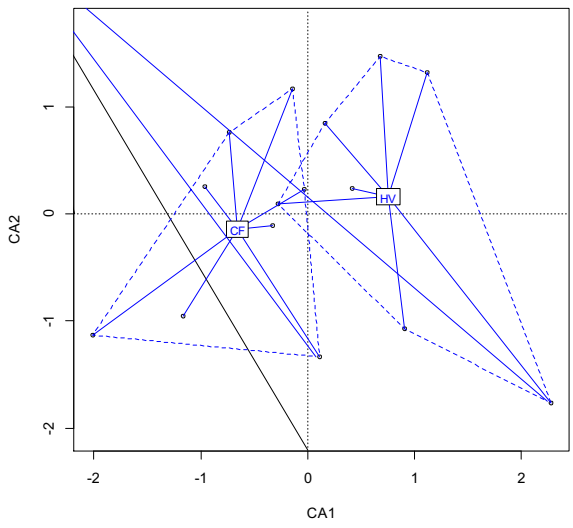

Figure 1. Multi-dimensional projection of soil samples by using a centroid analysis based on weighted Chi-square distances matrices of their 16S rRNA bacterial communities. The labels indicate the soil types, chemical fertilizer (CF) and hairy vetch (HV) soils.

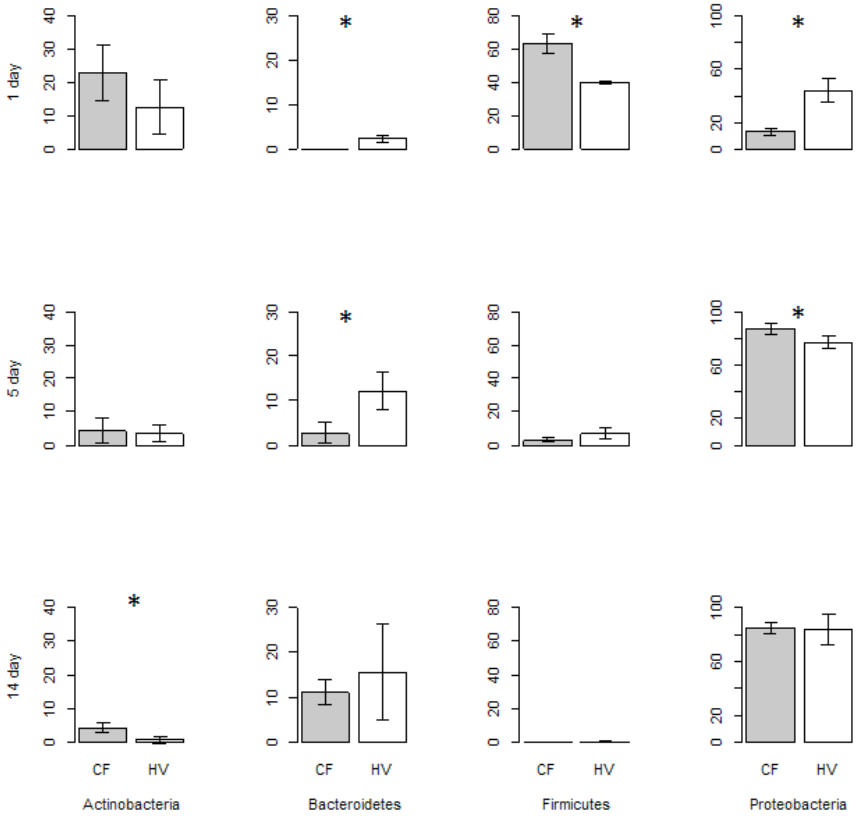

Figure 2. Short-term changes in dominated bacterial phyla in soils, with the history of chemical fertilizer (CF, grey bars) and of hairy vetch (HV, white bars), after the addition of excess C (as rice straw) and N (as nitrate) during an incubation experiment. The errors were standard deviations (n=3) and statistical significances between the soil types were shown using symbols (* $p<0.05$).

Discussion

Microflora composition in terms of long soil management

The HV soil was more dominated with bacteria than fungi. This result was opposite to other previous studies. Jeffery et al. (2010) showed that HV application increased the amount of fungi compared to the control soil in tomato cropping system. Similarly, Carrera et al. (2007) also indicated that HV application lead to high proportion of fungi compared to bacteria based on a phospholipid fatty acid analysis. These reciprocal results might be because of the different measurement method in terms of microbial analyses. We applied colony counting method and only culturable microorganisms were considered. However, studies referred above used other methods such as phospholipid fatty acid analysis thus they measured unculturable microbes. Thus, there is a possibility that we underestimated the abundance of microorganisms.

Based on the analyses of the similarity of the bacterial community structures of HV and CF, the two communities were significantly different (Fig. 1). Six phylla dominated both soils and there was a significant difference between the two soils in terms of the ratio of *Actinobacteria*, *Proteobacteria*, *Cyanobacteria* and *Gemmatimonadetes*. One of the dominant phyla, *Preteobacteria* is composed of several classes and response to carbon availability is different from each other (Fierer et al. 2007). Fierer et al. (2007) showed that the abundance of β-Proteobacteria was positively correlated with C mineralization rate thus β-Proteobacteria is considered to be more tolerate to high C availability. In this study, the ratio of β-Proteobacteria was significantly higher in HV soil (data is not shown). Thus, it is likely that the application of HV into soil increased C source in soil and might have increased the abundance of β-Proteobacteria.

Changes in soil nitrate concentrations and biomass C

Despite the clear difference in microbial community structures (Fig. 2), there was no significant difference in the decreases in NO_3^--N over time. Also, the biomass C was not clearly different between the two soils. This result was supported by a previous study that showed changes in microbial community composition were not

accompanied with its function (Buyer et.al 1997).

Response of bacterial community to C and N input

When the large amount of C and N sources were added into soil, differences between the microbial community structures in the two soils became smaller over time. The proportion of *Proteobacteria* markedly increased within 5 days after C and N addition (Fig. 2). Moreover, *Firmicutes* and *Bacteroidetes* which could not be detected in the original soil (before C and N addition) increased and became one of the dominant phyla during the incubation. *Proteobacteria* and *Firmicutes* are both described as fast-growing and stimulated in C rich environment (Bernard et al. 2007, Jemkins et al. 2010, Lee et al. 2011) and β-Proteobacteria and *Bacteroidetes* increased its abundance with high C availability, agreeing to our finding (Fierer et al. 2007). On the other hand, *Acidobacteria* which was reported to be able to survive with low level of nutrition (Fierer et al. 2007) disappeared when excess C and N sources were added into soil, despite its big abundance at initial condition (Fig. 2).

Conclusion

The long term (9 years) use of hairy vetch on a tomato greenhouse significantly changed the soil microbial community structures based on 16S rRNA analyses, increased the ratio of *Proteobacteria* and *Actinobacteria*, when compared to the soils at the same greenhouse without hairy vetch and with the use of N chemical fertilizer. However, the difference in the microbial community structures did not influence the N immobilization potentials, when C and N were applied to the soils. The addition of C and N significantly influenced the microbial community structures in the original soils. The differences in the community structures which were observed before the C and N addition became smaller. Therefore, we concluded that the long term use of organic source of N did not influence the soil's capacity to immobilize N, at least when excess C was applied.

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