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# The Impacts of Different Green Manure on Soil Microbial Communities and Crop Health

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**Abstract:** Green manure could improve soil nutrients and crop production, playing a significant role in sustainable agriculture. However, the impacts of green manure on crop health and the roles soil microbial communities play in the process haven't been clarified clearly yet. In this study, we investigated soil microbial community composition and structure in four tobacco farmlands, which were treated with different green manure (control, ryegrass, pea and rape), using 16S rRNA gene amplicons sequencing. Results showed that green manure had significant impacts on soil properties, microbial communities and tobacco health. First, soil total C, N and Ca content increased significantly in groups treated with green manure than control. Second, soil community diversity was significantly higher in groups treated with green manure. Third, green manure especially ryegrass, decreased tobacco disease (bacterial wilt) rate dramatically, and the process might be mediated by soil microbial communities. On the one hand, several microbial populations were found to be potentially disease inducible or suppressive. For example, the abundances of *Dokdonella* and *Rhodanobacter* were positively correlated to tobacco disease rate, while *Acidobacteira\_Gp4* and *Gp6* had negative correlations with tobacco disease. On the other hand, soil microbial communities were shaped by soil properties (e.g., pH, C and N content). In conclusion, our research showed that green manure could increase soil nutrients directly, and further improve tobacco health mediated by soil microorganisms, which may shed light on revealing interactions among soil properties, microorganisms and plants.

**Keywords:** green manure; soil microbial communities; crop health; Illumina sequencing

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## Introduction

Continuous cropping and inadequate replacement of nutrients removed in harvested materials or lost through erosion, leaching or gaseous emissions deplete fertility and cause soil organic matter levels to decline [1]. Thus, a large part of the increased yields of global cereal production resulted from great inputs of fertilizer. However, chemical fertilizer, industrially produced NH<sub>4</sub> and NO<sub>3</sub>, had negative impacts on environments and ecosystem, like the eutrophication of aquatic habitats, and increasing emission of gases that have critical roles in air pollution [2]. Meanwhile the

loss of nutrients necessitate the expense of increased fertilization [3]. In a word, traditional agricultural practices have negative impacts on environments and reduce the ability of ecosystems.

Green manure is a crop used primarily as a soil amendment and a nutrient source for subsequent crops, having great potential in increasing soil organic matter, pest control and increasing crop productivity [4]. It could increase soil organic matter, especially available nitrogen, and improve crop productivity (e.g., rice and wheat), and it was also reported that the concentrations of Ca, K, Mg, and Mn were higher in soils amended with green manure than synthetic fertility amendments [5, 6]. More importantly, soils in organic production systems lost less nitrogen into nearby water systems than did conventional production systems [7]. As for the influences of fertilizer on soil microbial organisms, it was reported that the amount of soil nitrogen in fields under conventional production systems has been negatively correlated with soil microbial components, whereas soil nitrogen in fields under organic production was positively correlated with soil microbial components [8]. For example, red clover manure increased and maintained higher bacterial and fungal biomass and high rates of enzyme (arylsulphatase) activity compared to control treated with chemical fertilizer [9]. Collectively, green manure or organic fertilizer was equally as well in improving soil nutrients and crop production as chemical fertilizer [10], but more environmentally friendly, playing a significant role in sustainable agriculture.

The impacts of green manure on crop disease are still controversial, probably due to that different manure had different impacts on soil microbial communities [11]. Soil-borne pathogens can be either actively suppressed by organic amendments or enhanced, depending on soil health conditions. A research demonstrated that green manure had no suppressive effect on *Pythium* (pathogenic to lettuce) population, and inferred that a strong increase in microbial activity may reduce N availability of crops [12]. The negative effects of green manure on crop health were verified in a study, that soil amendment with red clover green manure and compost increased the incidence of disease in wild mustard seedlings and dissolved organic carbon concentration was weakly correlated with disease rate [13]. However, there were also studies suggested that green manure treatments may achieve disease control by activating pathogen inhibitors (e.g., streptomycete), or by releasing allelochemicals generated during product storage or by subsequent microbial decomposition [14-16]. All in all, the issue needs to be explored further.

The complex relationship between soil microbial communities and plants, and the technical limitation in revealing composition and structure of microbial communities also hinder revealing relationship between green manure and crop health. The soil microbial community composition and structure are so sophisticated that many bacteria, which may affect tobacco health, still remain uncultured or unknown [17]. The development of metagenomic sequencing provides us an opportunity to explore the less abundant and primarily uncultured microbial communities. Although pyrosequencing analysis of 16S rRNA genes have been used to investigate the soil microbial communities, but few studies concern the impacts of green manure [18]. Studies in this area used isolates of soil-borne fungal pathogens or disease inhibitors to perform in vitro pathogen inhibition assays, ignoring potential disease inducible or suppressive microbial populations [14, 15]. In this study, Illumina MiSeq sequencing of 16S rRNA genes was used, which has been demonstrated to be an effective technology in capturing the less abundant taxa [19]. We investigated the impacts of different green manure on soil properties, microbial communities and tobacco health,

in order to (i) find the most effective green manure in controlling tobacco disease; (ii) clarify the impacts of green manure on soil properties and microbial communities; (iii) explore the relationship among green manure, soil microbial populations and tobacco health.

## Materials and Methods

### Site description and sampling

As a large scale investigational study, four farmlands with similar soil type were selected. They were located in Xiangxi, Hunan province. Tobacco had been cultivated under different green manures, which were control (no manure), ryegrass, pea, and rape. Ten samples were collected from each field using checkerboard sampling method on July 28<sup>th</sup> (tobacco mature stage) with the permission of the owners. A hole puncher (3cm in diameter, 20cm in depth) was used to collect soils between tobacco plants. Each soil sample was about 400g. The number of tobacco plants with bacterial wilt was counted to calculate tobacco disease rate of a farmland according to the disease symptom described before [20]. Water content was measured by drying 5g of each soil sample in a dry oven for 24 hours at 98°C. Soil pH was measured by a pH measure, and samples were dissolved in sterile water (5g/25mL) before pH measure. Geochemical properties of soil were analyzed by ICP-AES [21]. Total organic N was quantified by Kjeldahl distillation [22]. The amount of total organic C was analyzed by potassium dichromate oxidation-ferrous sulphate titrimetry [23].

### DNA extraction, amplification and sequencing

After mixing separately, 0.5g of each soil sample was used for DNA extraction. DNA was extracted using a MO BIO PowerSoil DNA Isolation Kit (MO BIO, 12888-100) with grinding and SDS lysis followed by gel purification, following the classic protocol at <http://ieg.ou.edu/protocol.htm>. The V4 region of the 16S rRNA genes were amplified with the primer pair 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GACTACHVGGGTWTCTAAT-3') combined with Illumina adapter sequences, a pad and a linker of two bases, as well as barcodes on the reverse primers [24]. Sample libraries were generated from purified PCR products. The MiSeq 500 cycles kit was used for 2x250 bp paired-ends sequencing on MiSeq machine (Illumina, San Diego, CA).

Sequences with perfect matches to barcodes were split to sample libraries, and were trimmed using Btrim with threshold of QC higher than 20 over five bp window size and the minimum length of 100 bp [25]. Forward and reverse reads with at least 10 bp overlap and lower than 5% mismatches were joined using Flash [26]. After trimming of ambiguous bases (i.e., N), joined sequences with lengths between 240 and 260 bp were subjected to chimera removal by U-Chime [27]. OTU clustering was through UCLUST at 97% similarity level [28], and taxonomic assignment was through RDP classifier [29] with a minimal 50% confidence estimate. The above steps were performed through the Galaxy pipeline (<http://zhoulab5.rccc.ou.edu/>). Subsequent analyses were performed in R [30]. Singletons were removed for downstream analyses. Samples were rarefied at 18,000 sequences per sample. All the 16S rRNA gene sequences were deposited in GenBank database and the accession number were KR831285 - KR855564. Dissimilarity tests were based on Bray-Curtis dissimilarity index using analysis of similarities (ANOSIM) [31]. Differences in abundances across metal concentration gradient were determined by a one-way analysis of variance (ANOVA) followed by Least Significant Difference (LSD) test

[32]. Bacterial community diversity was calculated using Shannon-Weiner's diversity (H') and evenness. Multivariate statistical analyses of sequencing data were conducted, including detrended correspondence analysis (DCA) for comparing the different functional gene communities. Mantel test [33] and partial least squares path modeling (PLSPM) were used to calculate the correlations between bacterial abundance and environmental attributes. All the analyses were performed in R v. 2.6.1 with the packages *vegan* and *plsrm* (v. 1.11-3) [30] or online (<http://ieg.ou.edu/>).

## Results

### Characterization of tobacco health and soil properties

A summary of soil properties, including soil pH, water content and amount of elements (e.g., Ca, C, N), was described in Table 1. Water content was significantly ( $p < 0.05$ ) higher in Control. All soil samples were acidic with pH ranged from 5.10 to 5.76, and higher in Rape and lower in Control and Ryegrass. Soil composition of four groups was also different. For example, the content of Ca, C and N were significantly ( $p < 0.05$ ) lower in Control than in other groups. Control had the highest tobacco disease rate (80.45%), while disease rate was the lowest in ryegrass treated group (3.38%) (Table 2).

Table 1. Soil properties in fields treated with different green manures

Sample	PH	Water content (%)	Total N (%)	Total C (%)	Ca (ppm)	Fe (ppm)	Co (ppm)	Zn (ppm)
Control	5.10±0.20	17.07±1.48	0.096±0.008	0.852±0.067	1580.63±264.78	15277±1274.81	3.91±2.37	42±5.93
Ryegrass	5.35±0.22	14.42±3.81	0.125±0.09	1.026±0.303	2769.60±481.29	15660±1858.47	4.89±2.60	44.80±7.46
Pea	5.45±0.37	14.25±0.75	0.12±0.01	1.106±0.349	2653.40±792.17	15475.3±1885.15	3.84±2.70	44±9.44
Rape	5.76±0.53	15.62±2.18	0.104±0.003	0.950±0.084	2473.70±837.27	15805.5±3105.41	3.75±3.38	46.2±10.30
ANOVA	a,a,ab,b	a,b,b,ab	a,b,c,d	a,ab,b,ab	a,b,b,b	a,a,a,a	a,a,a,a	a,a,a,a

\* Significant differences ( $p < 0.05$ ) among four groups are indicated with alphabet.

Table 2. Diversity of microbial communities of four groups and tobacco disease rate

	Control	Ryegrass	Pea	Rape
Shannon diversity	6.637514 (a)	6.923182 (b)	6.992128 (b)	6.658385 (ab)
Pielou evenness	0.833277 (a)	0.85387 (b)	0.86093 (b)	0.833226 (a)
Chao value	10193.43	12107.86	11800.48	12233.25
Disease rate	80.45%	3.38%	8.78%	22.97%

\*Significant ( $p < 0.05$ ) differences among four groups are labeled with alphabet.

### Overview of microbial community diversity

After resample, we obtained 18000 high-quality 16S rRNA gene sequences per sample. Rarefaction curve showed that the numbers of OTUs were almost saturated in all samples, and enough for community analysis (Figure S1). After clustering at 97% sequence identity, 14,629 OTUs were identified in this study, out of 127 OTUs were classified as archaea. In domain archaea, 62.28% of reads were classified as *Fervidicoccus*, and 26.21% were unclassified genera of phylum *Euryarchaeota*. Within the bacterial domain, four major microbial phyla accounted for 55.79% to 65.39% of all reads, and they were *Proteobacteria* (21.69% ~ 41.44%), *Chloroflexi* (2.78% ~ 16.24%), *Acidobacteria* (13.05% ~ 23.62%), and *Actinobacteria* (4.13% ~

8.13%). And about 11.78% to 22.65% of sequences were not assigned to any known phylum (Figure S2). Shannon diversity index and Pielou evenness index were significantly ( $p < 0.05$ ) lower in Control (6.637, 0.833) and Rape (6.658, 0.833), but higher in Ryegrass (6.923, 0.854) and Pea (6.992, 0.861) (Table 2).

To evaluate the difference of these microbial communities in structure generally, we conducted dissimilarity test and DCA. Dissimilarity test showed that microbial communities were significantly ( $p < 0.01$ ) different among four groups (Table S1). Similar results can also be seen from DCA graph, which showed that samples of each group were well separated, except for that several samples of Rape were clustered with samples of Control (Figure 1).

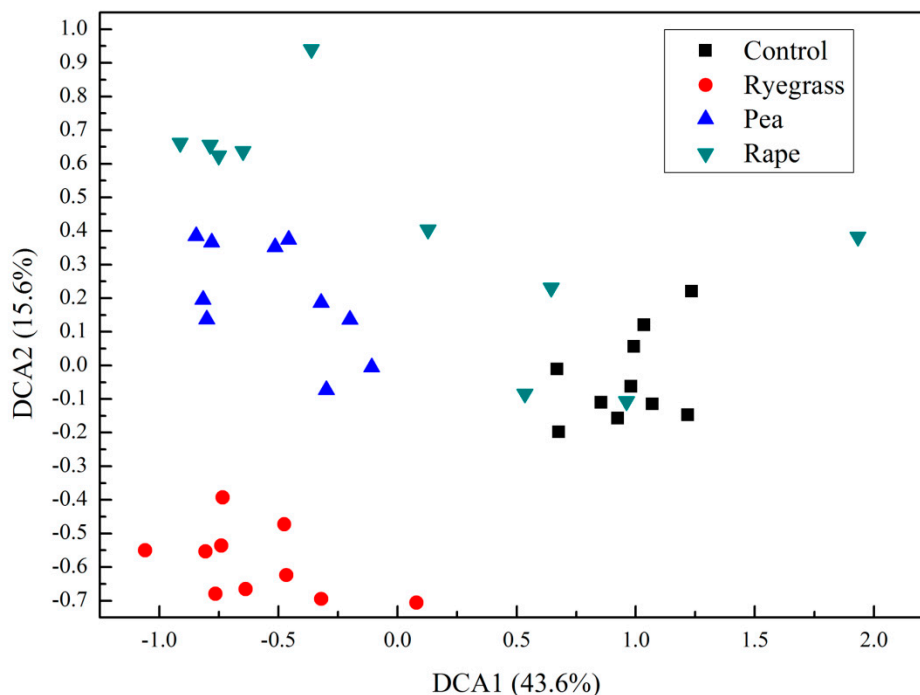


Figure 1. Detrended correspondence analysis (DCA) of 16r RNA gene sequencing data

### The impact of different green manure on soil microbial communities

Fertilization of green manure had significant impacts on soil microbial community composition and structure. At the phylum level, *Proteobacteria*, *Actinobacteria* were more abundant in Control; *Crenarchaeota* was more abundant in Rape; *Acidobacteria* was more abundant in Pea and had the fewest reads in Control; *Chloroflexi* and *Planctomycetes* were more abundant in Ryegrass. At the genus level, *Acidobacteria\_Gp6*, *Acidobacteria\_Gp4* and *Gp16* were more abundant in Pea; *Spartobacteria\_genera\_incertae\_sedis*, *Gemmatimonas*, *Dokdonella* and *Rhodanobacter* were more abundant in Control (Figure 2). Among microbial populations that were reported to be related to tobacco health, *Ralstonia* was significantly more abundant in Control; *Pseudomonas* was less abundant in Ryegrass; and *Bacillus* was more abundant in Control and Pea.

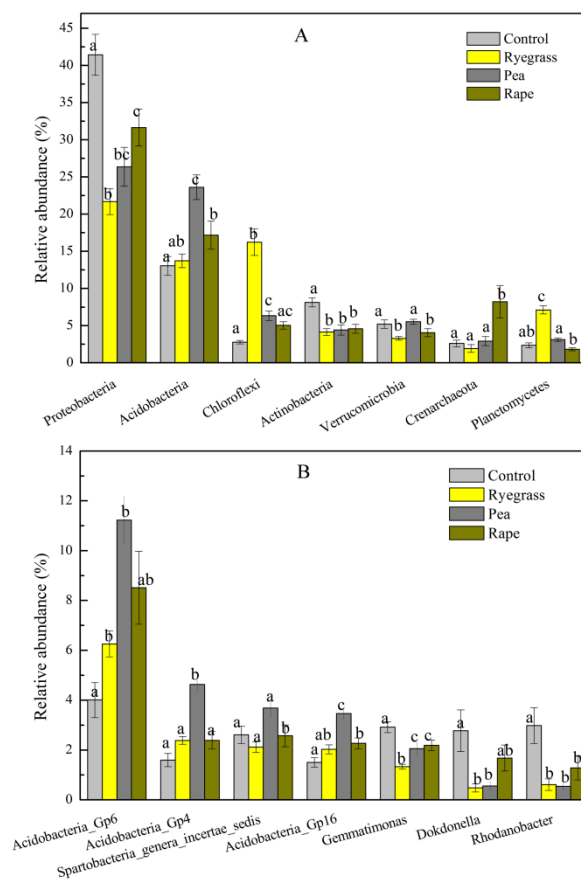


Figure 2. Relative abundances of major microbial populations in four groups at the phylum level (a) and the genus level (b). Significant differences ( $p < 0.05$ ) among four groups are labeled with alphabet.

### Correlations among soil properties, microbial communities and tobacco health

Mantel test was conducted to evaluate impacts of soil properties on microbial communities, and results showed that soil water content, pH, and the amount of Ca, Fe, C, N influenced soil microbial communities significantly ( $p < 0.05$ ) (Table S2). Furthermore, relationship between specific microbial population and environmental factor was analyzed using Pearson correlation. Seven phyla (e.g., *Proteobacteria*, *Actinobacteria*) were found to be negatively correlated with soil Ca content, while *Acidobacteria* and *BRC1* were positively correlated with Ca content. Eight phyla (e.g., *Proteobacteria*, *Actinobacteria*) were negatively related to N. And four phyla (e.g., *Chloroflexi*, *Planctomycetes*) were positively correlated to N. Seven genera (*Acidobacteria\_Gp4*, *Acidobacteria\_Gp6*) were positively correlated to Ca, ten genera (*Dokdonella*, *Rhodanobacter*) negatively. Eight genera were positively correlated to N, 12 genera (*Dokdonella*, *Rhodanobacter*) were negatively correlated. *Pseudomonas* and *Bacillus* were negatively correlated with N (Table S3).

Both soil properties and microbial populations were related to tobacco disease rate. The abundances of four major (relative abundance > 0.6%) phyla (*Proteobacteria*, *Actinobacteria*, *Gemmatimonadetes* and *Nitrospira*) and eight genera (e.g., *Dokdonella*, *Rhodanobacter*) were positively correlated to tobacco disease rate. The abundances of four phyla (*Acidobacteria*, *Chloroflexi*, *Planctomycetes* and *WS3*) and eight genera (e.g., *Acidobacteria\_Gp6*, *Acidobacteria\_Gp4*) were negatively correlated to tobacco disease rate. Also, soil Ca, N and C contents were negatively

correlated to tobacco disease rate, while water content, Ammonium N, rapidly available P and rapidly available K were positively correlated to tobacco disease rate. Almost all microbial populations that positively correlated with tobacco disease rate were negatively correlated with N and Ca amount (Table S3). Collectively, PLSPM was constructed to profile the relationship among soil properties, microbial communities and tobacco disease. Results showed that soil properties had impacts on soil microbial communities; both soil properties and microbial communities contributed to tobacco disease (Figure 3). All the correlations were significant ( $p < 0.01$ ) in the model. Goodness of fit value was 0.4405, bigger than 0.35, indicating that the model was reliable.

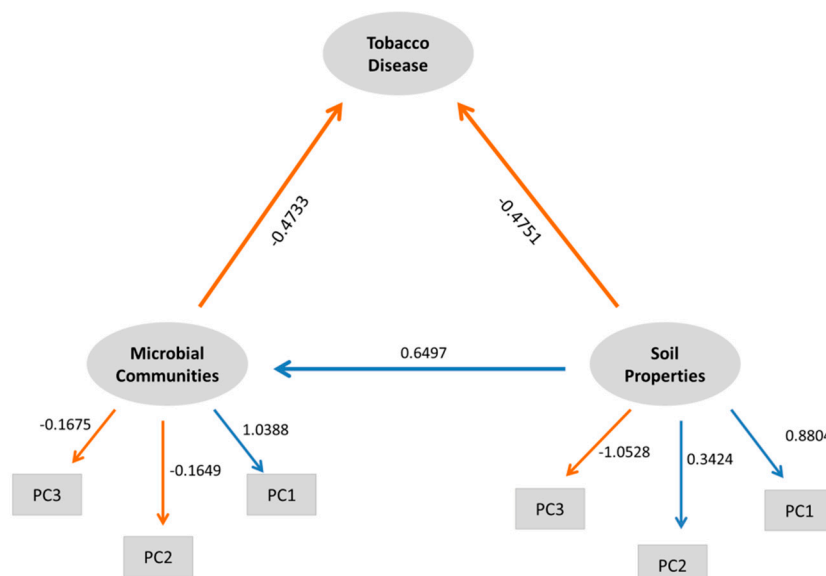


Figure3. Partial least squares path modeling (PLSPM) about correlations among soil properties, microbial communities and tobacco disease

## Discussion

As the potential replacement of chemical fertilizer, green manure could improve soil nutrients and crop production, but its impacts on crop health haven't been clarified clearly yet [4, 11]. In order to explore the impacts of green manure on tobacco health, and reveal relationship among soil properties, microbial communities and plant disease, 16S rRNA gene sequencing and PLSPM were used in this study. Our analyses showed that all three kinds of green manure could increase soil organic matter, increase soil microbial community diversity and decrease tobacco disease rate compared to the control. And the effects were slightly different among three groups, as ryegrass treated group had the lowest tobacco disease rate. It has been realized that the effect of each green manure on soil properties and crop yield depends upon its chemical composition [34]. Green manure of ryegrass, pea and rape had different composition, thus might influence soil properties in different ways. Tobacco, pea and rape are all dicotyledons, while ryegrass is monocotyledon. Monocotyledon and dicotyledon usually had different nutrient requirements, root exudates and microbial pathogens [35]. For instance, phosphorus mobilized by a dicotyledon (faba bean)

increases the growth of a kind of monocotyledon (maize) grown in an intercropping system [36]. Similarly, we could speculate that ryegrass manure might affect soil properties and microbial communities differently, resulted from its distinct composition. Not only the most effective green manure in this study, ryegrass was also used as effective green manure for many other crops, such as wheat, maize and potato, due to reducing N leaching loss over winter and improving N supply [15, 37], indicating its potential in sustainable agriculture.

First of all, soil properties were affected by green manure, and the changed soil properties were beneficial for tobacco growth. Previous research showed that organic fertilizer greatly increased soil organic C, total N, P and K, available N, P and K content [38]. In this study, samples treated with green manures had higher Ca, N and C content. There is no doubt that increased nutrient is beneficial for plant growth and activity of soil microorganisms. But the importance of Ca hasn't been clarified sufficiently before. Ca is a necessary element for plant growth, thus may increase tobacco's resistance to pathogen indirectly. Moreover, Moshe Sagi found that the plant homolog can be stimulated directly by  $\text{Ca}^{2+}$  to produce  $\text{O}_2^-$ , which was considered to be a component of the resistance response of plants to pathogen challenge [39]. Therefore, the increased Ca content by green manure might be key factors in suppressing tobacco disease.

Then, soil microbial communities were influenced by soil properties. It was commonly acknowledged that bacterial and eukaryotic community structures were significantly affected by soil properties, especially C and N content [40]. Our results showed that soil microbial community composition and structure were different among four groups, and community diversity was higher in groups treated with green manure. We speculated that the increased biodiversity resulted from the increased soil nutrients. And it has been reported that green manure had positive influence on soil biomass, which was consistent with our result [4]. As for the meaning of the increased biodiversity, there was no direct relationship between biodiversity and function [41], but the decreased biodiversity impair function and productivity of the ecosystem, because the ability of ecosystems to provide some services depends on both the number and type of species in an ecosystem [42, 43]. So soils with the higher biodiversity were more resistant to the stress. In this way, green manure increased biodiversity of tobacco farmlands, and might make them more resistant to infection of pathogen.

Specifically, soil microbial community composition and structure changed significantly among four groups. And the abundance of *Raltonia*, which were conducive to tobacco bacterial wilt, was higher in control [44]. A previous study showed that green manure decrease tobacco disease rate by decreasing the amount of *Raltonia* and increasing the abundance of *Bacillus* using qPCR [45]. Basically, it was consistent with our result, that green manure could control pathogen populations. However, *Pseudomonas* and *Bacillus* had no significant negative correlation with tobacco disease rate, despite that they showed great potential in disease suppression [46]. For instance, *Pseudomonas fluorescens* could produce antifungal phloroglucinols in soils naturally suppressive to tobacco black root rot and reduced the extent of black root rot from 82% to 28% [47]. The reason could be that increasing the amount of probiotic bacteria was not the only way to improve tobacco health. For example, organic fertilizer could alleviate allelopathic autotoxicity of continuous cropping tobacco [48].



And the abundance pattern of other microbial populations might indicate their relationship with tobacco health. For example, the abundance of *Acidobacteria* was negatively correlated with tobacco disease rate, suggesting their potential in disease control. *Acidobacteria* is an acidophilic chemoorganotrophic bacterium, and it was reported that many sequences involved in nitrogen fixation and carbon cycling were founded to be associated with *Acidobacteria* [49, 50]. *Acidobacteria* also had genes that encode polyketide synthase and nonribosomal peptide synthase enzymes, which well known for their roles in the synthesis of siderophores, as well as other natural products such as antibiotics, antifungals, and antinematodal agents [51]. In this way, *Acidobacteria*, especially *Gp4* and *Gp6*, probably play important roles in tobacco disease control. By contrast, the abundance of *Proteobacteria*, mainly composed of *Dokdonella* and *Rhodanobacter*, was positively correlated with tobacco disease rate. *Dokdonella* and *Rhodanobacter* are aerobic, chemo-organotrophic genera of *Xanthomonadales* order, which contains many plant pathogen species [52, 53]. Also, *Rhodanobacter* had the capability of denitrification, and might compete nitrogen source with tobacco [54]. In a word, no plant beneficial properties (e.g., nitrogen fixation) were identified in them, and they were potential nutrient competitor and disease inducer.

Finally, green manure, soil properties and microbial communities shaped tobacco health together. It has been realized for a long time that soil properties and microorganisms interact with each other, and both of them influencing plant growth and health [37, 45, 48]. Our study explored the interactions under the impacts of different green manure, as all of them changed significantly with green manure type. And their different change pattern indicated their relationship. For example, Almost all microbial populations that positively correlated to tobacco disease rate (e.g., *Dokdonella* and *Rhodanobacter*) were negatively correlated with soil total organic N and Ca content; while microbial populations that negatively correlated to tobacco disease rate (e.g., *Acidobacteria\_Gp4*, *Acidobacteria\_Gp6*) were usually positively related with contents of soil organic N and Ca. The result suggested that green manure increased soil nutrients, which might improve tobacco health directly or indirectly through increasing the amount of probiotic bacteria.

## Conclusions

In summary, we found that: (i) green manure increased soil nutrition, including N, C and Ca content; (ii) green manure had significant impacts on diversity, composition and structure of soil microbial communities; (iii) green manure decreased tobacco disease rate, which was mediated by soil properties and certain microbial populations.

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**Author Contributions:** Conceived and designed the experiments: Huaqun Yin and Wu Chen; performed the experiments: Yabing Gu and Hongwu Yang; Analyzed the data: Hongwu Yang, Jiaojiao Niu and Jiemeng Tao; contributed to sample collection: Chao Zhang and Siyuan She; Wrote the paper: Hongwu Yang; Revised the paper: Hongqi Yang.

**Conflict of interest:** The authors declare that they have no conflicts of interest.

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