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Natural Farming Improves Soil Quality and Alters Microbial Diversity in a Cabbage Field in Japan

Jianli Liao ¹, Qicong Xu ², Huilian Xu ^{2,*} and Danfeng Huang ^{1,*}

¹ Key Laboratory of Urban Agriculture (South), Ministry of Agriculture, School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai 200240, China; Jerry0427@sjtu.edu.cn

² International Nature Farming Research Center, Matsumoto, Nagano 390-1401, Japan; qicongx@163.com

* Correspondence: huilian_xu81@163.com (H.X.); hdf@sjtu.edu.cn (D.H.); Tel.: +86-21-3420-6943 (D.H.)

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Abstract: Natural farming (NF), an environmentally friendly agricultural practice similar to organic farming, was developed in Japan. Unlike conventional farming, little is known about the influence of NF on soil microbial communities, especially the surface soil. We therefore compared the effect of seven years' conventional practice (CP), conventional practice without chemicals (CF), and NF on soil properties and microbial community structure at two soil depths (0–10, 10–20 cm) in an experimental cabbage field. Both soil depth and agricultural practice significantly influenced edaphic measures and microbial community structure. NF improved bulk density, pH, electrical conductivity, urease activity, and nitrate reductase activity in topsoil; similar trends were observed in deeper soil. Pyrosequencing demonstrated that the use of pesticides in conventional farming (CP) led to lower microbial abundance and diversity in topsoil than CF. Similarly, NF increased microbial abundance compared to CP. However, distinct taxa were present in the topsoil, but not deeper soil, in each treatment. CP-enriched microbial genera may be related to plant pathogens (e.g., *Erwinia* and *Brenneria*) and xenobiotic degraders (e.g., *Sphingobacterium* and *Comamonas*). The microbial community structure of NF was distinct to CP/CF, with enrichment of *Pedomicrobium* and *Solirubrobacter*, which may prefer stable soil conditions. Network analysis of dominant genera confirmed the more stable, complex microbial network structure of the 0–10 cm than 10–20 cm layer. *Flavisolibacter/Candidatus Solibacter* and *Candidatus Nitrososphaera/Leuconostoc* are potentially fundamental taxa in the 0–10 cm and 10–20 cm layer networks, respectively. Overall, we show that NF positively affects soil quality and microbial community composition within sustainable farming systems.

Keywords: agricultural practice; sustainability; bulk soil; bacterial community structure; pyrosequencing; natural farming

1. Introduction

Over the last half century, the green revolution has significantly increased agricultural production through the use of fertilizers and agrochemicals, and thus helped to feed the growing population of the world [1]. However, agricultural intensification mainly involves indiscriminate use of chemical fertilizers and agrochemicals and has contributed to negative environmental issues such as global warming, eutrophication of water systems, and soil degradation. Soil is a key limiting component of agroecosystems that cannot be easily or quickly renewed. Moreover, soil has major effects on plant growth as it regulates processes such as organic material degradation, nutrient cycling and adsorption, and decomposition of xenobiotics [2], and soil quality is highly related to sustainable crop production.

Strategies to improve soil health and fertility, conserve soil resources, and establish a sustainable agricultural model, such as no tillage, controlled traffic farming, and organic farming, have recently received growing attention. These farming management techniques have been shown to reduce soil

compaction, soil erosion, maintain organic matter, and enhance soil biodiversity, which are beneficial to soil conditions [3–5]. Of these practices, organic farming is the most widely adopted by farmers; 44 million hectares are reported to be managed organically worldwide [6]. Other sustainable farming systems were proposed long ago. For example, Mokichi Okada (1882–1955) proposed the concept of natural farming, which is similar to organic farming, as an alternative to conventional farming in Japan in 1935. The principles of natural farming are focused on environmental biodiversity and soil health and follow the laws of nature in agricultural production [7].

Several natural farming management strategies have been developed, including crop rotation, intercropping, reduced tillage, mulching with plant residue, avoiding the use of agrochemicals, and application of biofertilizers. These practices are similar to the organic farming practices conducted in other countries that have been proposed as a more sustainable method of agricultural production than conventional farming [8–11]. Several studies have confirmed that natural farming can improve soil properties, biodiversity, and enzyme activity within different agroecosystems (e.g., vegetable and rice) in Japan [7], but little is known about the composition and characteristics of the soil microbial communities in natural farming systems.

Soil microbes play pivotal roles in a variety of soil processes [9]. For example, soil microbes participate in nutrient processing by promoting the decomposition of organic materials [12], are involved in degradation of xenobiotics [13] and soil carbon sequestration [14], and even protect against crop diseases [15,16]. Several comparisons of organic and conventional farming systems have indicated agricultural practices significantly impact the soil microbial community [5,8–11]. Most of these studies indicated microbial abundance and diversity were higher in organically farmed soils than conventionally cultivated soils [5,11]. Specifically, the differences between organically and conventionally managed soils could be generally attributed to the management processes, including the fertilization strategy, tillage regime, plant residue management, crop rotation system, and pest and disease control schemes [17]. Previous studies indicated that application of organic fertilizer increased soil microbial richness and diversity compared to application of a mineral fertilizer; this effect was mainly attributed to increased levels of copiotrophs through introduction of bioavailable organic substances [8], rather than exogenous microbes [18,19]. Furthermore, combined chemical and organic fertilization was shown to reduce the abundance of soil-borne pathogens (e.g., *Fusarium oxysporum*) and increase beneficial microbes (e.g., *Bacillus* spp. and *Trichoderma* spp.) compared to chemical fertilization, and the authors suggested these effects could further suppress or prevent disease [15]. Conventional tillage generally plows the surface soil (0–20 cm), which increases soil aeration, stimulates the release and degradation of soil organic matter, and, as a consequence, promotes nutrient cycling and increases soil microbial diversity compared to reduced tillage [20]. In terms of plant residue management (e.g., removal, incorporation with soil, or mulching the soil surface), straw mulch significantly increases soil microbial biomass and activity by increasing the availability of carbon and water compared to non-mulched soil [21]. Furthermore, a meta-analysis indicated crop rotation increases soil microbial abundance and diversity compared to continuous cropping [22]. Another study indicated soil under monoculture had a lower abundance of antagonists (e.g., *Trichoderma* spp.) than organically managed soil [23]. Therefore, these observations suggest that agricultural practices alter the soil microbial diversity and also affect the abundance of plant pathogens or beneficial microbes, implying proper management could help to establish a beneficial microbial community to maintain or improve the soil environment and increase plant productivity [8].

The surface soil layer (0–20 cm) is regarded as the hot-spot of anthropogenic activities within agricultural systems. The majority of studies on the impact of agricultural practices on microbial community composition and diversity have predominantly focused on this horizon (0–20 cm) [8,9,11,20,23]. However, some agricultural practices, such as reduced tillage and application of chemicals to topsoil, can lead to minor disturbances in the deeper soil. Hence, samples collected from the 0–20 cm soil layer may not be typical of the corresponding cultivation systems. Furthermore, little is known about the variations in microbial community structure in the surface soil at different depths across different

agricultural practices. In addition, while substantial information is available on the soil microbial communities within a variety of crop agroecosystems, such as wheat [20], rice [24], and soybean [25], the soil microbial communities within vegetable production systems remain poorly understood [9].

In this study, we measured edaphic properties and used high-throughput sequencing of 16S rRNA gene markers to examine the microbial community response at two different soil depths (i.e., 0–10 cm and 10–20 cm) in an experimental cabbage field with plots under three different agricultural managements, namely conventional practice with chemicals (CP), conventional practice without chemicals (CF), and natural farming (NF). The objective of this study was to obtain a better understanding of how agricultural practices influence soil microbial community composition and preferential taxa at two soil depths in different cultivation systems. We hypothesized that long-term natural farming would improve soil quality and also microbial abundance and diversity compared to conventional farming.

2. Materials and Methods

2.1. Experimental Site

This investigation was conducted at an experimental field established in 2009 at the International Natural Farming Research Center (INFRC), Matsumoto, Japan (geographical position 34.4° N, 137.5° E). This site experiences a subtropical climate with ~1000 mm annual precipitation and is at an altitude of 686 m. The soil is Andosols according to the Food and Agricultural Organization (FAO) classification [26].

Three cultivation systems were examined in this study: (1) conventional practice with chemicals (CP); (2) conventional practice without chemicals (CF); and (3) natural farming (NF), as shown in Table 1. Each management system was practiced within a 7.2 m × 22.8 m plot. The fertilization regime for the CP plot was as recommended by the Japanese Agricultural Cooperative in Matsumoto. First, chemical fertilizer (N-P-K content, 14–11.2–5.6 kg ha⁻¹) was spread and incorporated by ploughing to a depth of approximately 15 cm. Cabbage seedlings were transplanted into the field (crop density: 4160 plants ha⁻¹) at the end of July. Ten days after transplantation, top dressing fertilizer was applied at a rate of 7.2–1.6–3.2 kg ha⁻¹. Commercial pesticides (acetamiprid, fluidireidium, cartap hydrochloride, benzoic acid salt, and chlorfenapyr) and bioagents (*Bacillus subtilis*) were also used in the CP plot for disease and pest control. There was only one crop season (i.e., cabbage in autumn) per year in the CP plot. Agricultural practices and fertilization were the same for the CF and CP plots, though the pest and disease control practices were not applied on the CF plot.

Table 1. Agricultural practices applied in the three cultivation systems.

Practice	CP	CF	NF
Fertilizer type	Chemical fertilizer	Chemical fertilizer	Bioorganic fertilizer
Inputs (kg ha ⁻¹ y ⁻¹)			
N	21.2	21.2	7.2
P	12.6	12.6	6.84
K	8.8	8.8	1.8
Tillage	Ploughing with 15 cm depth	Ploughing with 15 cm depth	Reduced-tillage with 5 cm depth
Pest and disease control	Agrochemicals and bioagent	None	None
Rotation system	Cabbage	Cabbage	Cabbage-Rye
Residue mulch	None	None	Rye straw

Note: CP, conventional practice with chemicals; CF, conventional practice without chemicals; NF, natural farming practice.

The NF practices included crop rotation, application of biofertilizer, reduced tillage, and mulching with plant residue, as previously described [7]. The rotation system for the NF plot was rye (*Secale cereale*) in winter and cabbage (*Brassica oleracea* L. cv. Shoshu) in autumn. After harvesting the rye in the first week of July, the remaining above-ground parts were removed and incorporated with 180 kg ha⁻¹ biofertilizer (4.0–3.8–1.0) into the top 5 cm of surface soil. The biofertilizer was produced by anaerobic fermentation of soybean meal, rice bran, fish meal, molasses, and effective microbes for at least 3 weeks [7]. Ten days after transplantation, 80 kg ha⁻¹ biofertilizer was mixed with the top 1 cm of

surface soil as a top dressing. The rye straw was cut to 5 cm and applied as a mulch on the NF plot to maintain the soil water content and temperature and prevent weed growth. No biocontrol agent was applied to the NF plot. The cabbage seedling transplantation and cropping times were the same for all agricultural practices. In addition, furrow irrigation was applied simultaneously on all three plots to maintain the soil moisture content when required.

Soil pH, EC, total C, and total N of this experimental site were 6.26, 0.10 mS cm⁻¹, 5.07%, and 0.38% at the 0–10 cm depth in 2009, respectively. The dynamic changes in these soil chemical properties under different managements are shown in Figure S1.

2.2. Soil Sampling and Analysis

Soil samples (from depths of 0–10 and 10–20 cm) were collected from each plot using a stainless-steel auger after cabbage harvesting in October, 2016. All samples were taken no less than 15 cm away from cabbage stalks, and plant residue was removed from the soil surface before sampling. In total, 15 samples were collected from each plot and depth. After sampling, the soil samples were stored in polyethylene bags and kept on ice until transportation to the laboratory. Groups of three samples were randomly picked for each cultivation system and depth, mixed thoroughly, and then passed through a sterilized 2-mm sieve as one replicate, resulting in five replicates per cultivation system and depth. Each replicate sample was divided into three parts: one was air-dried and passed through a 2-mm sieve and another was stored at 4 °C for subsequent soil analysis, and the other was stored at –80 °C prior to molecular analysis.

Soil pH and electrical conductivity (EC) were assessed after resuspending 10 g air-dried soil in 50 mL deionized water and stirring manually three times within 30 min [27]. Total C and N were measured by dry combustion of air-dried soil using a CN corder (MT-700, Yanoco, Kyoto, Japan). The core method was used to measure the soil water content and bulk density [28]. NO₃⁻-N, NH₄⁺-N, urease activity and nitrate reductase activity were measured in fresh soil. NO₃⁻-N and NH₄⁺-N were quantified using the Kjeldahl method [29] after extraction in 1 M KCl (1:5, *m/v*) [30]. Urease activity was determined following a previously described method [31]; the content of NH₄⁺-N released when soil samples were incubated with borate buffer (pH 10) containing and lacking urea was assessed. Nitrate reductase activity was determined as the reduction of NO₃⁻-N into NO₂⁻-N [32].

2.3. Soil DNA Extraction, Sequencing, and Processing

Soil DNA was extracted from 0.5 g wet soil using E.Z.N.A. Soil DNA Kits (Omega Bio-Tek, Norcross, GA, USA) following the manufacturer's instructions. DNA quality and concentration were assessed using a NanoDrop 2000 fluorospectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Soil DNA was subsequently purified and sequenced by Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China). The 515F/907R primer set was used for amplification of the V4-V5 regions of the microbial hypervariable 16S rRNA genes on an Illumina Miseq sequencer. All sequence data has been deposited in the National Centre for Biotechnology Information Sequence Read Archive (SRA) under accession no. PRJNA506287. Quality control and annotation of the raw sequences were performed according to our previous study [5].

Raw sequences were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME™, version 1.8.0) software package [33]. Quality filtering was implemented to discard low-quality reads with an average Phred score lower than 20, under 150 bp in length, or sequences containing ambiguous N bases and mismatched primers. Fast Length Adjustment of Short Reads (FLASH) software (v1.2.7) was used to assemble remaining paired-end reads [34]. Operational taxonomic units (OTUs) were classified based on these contigs at 97% sequence identity using UCLUST [35]. A representative sequence from each OTU was aligned against the Greengenes database (Release 13.8) using BLAST [36]. Finally, OTUs accounting for more than 0.001% of all sequences were subjected to further analysis.

2.4. Statistical Analysis

Both multivariate and univariate analyses were used to examine the overall effects of soil depth (i.e., 0–10 and 10–20 cm) and cultivation system (i.e., CP, CF, and NF) on soil properties and microbial community structure. For multivariate analysis, unconstrained principal coordinate analysis (PCoA) was conducted to visualize the similarities between these two factors (soil depth \times cultivation system) based on a Euclidean distance of normalized soil variables in the R environment (3.4.1) using the vegan package. Permutational multivariate analysis of variance (PERMANOVA) with this distance matrix was then used to estimate the significance of soil depth and cultivation system over 9999 permutations with PAST3 software [37]. For univariate analysis, the significance of the differences among treatments were compared using Duncan's new multiple range test; p -values < 0.05 were considered significant.

To assess microbial community structure, alpha diversity indices—including the rarefaction curve, Good's coverage and the Chao1, abundance-based coverage estimator (ACE), Shannon and Simpson indices—were generated by QIIME and compared using Duncan's test ($p < 0.05$). Microbial community beta diversity in each treatment was analyzed using unconstrained PCoA based on Bray–Curtis index values. PERMANOVA was then applied to this distance matrix to evaluate the significance of the effects of soil depth and cultivation system on microbial community structure. Preferential microbial taxa were compared between different cultivation systems at the same soil depth using linear discriminate analysis (LDA) effect size (LEfSe, LDA score > 3.0) [38] and visualized at the phylum and genus levels using Microsoft Excel. Subsequently, strong ($r > 0.6$ or $r < -0.6$), significant ($P < 0.01$) correlations between the 50 most abundant genera in the three cultivation systems at each soil depth were determined by Spearman's rank correlation analysis. These correlations were used to create a microbial network in Cytoscape 3.2.1 [39] and further generate network topological properties such as node (genus) number, edge (strong interaction) number, network diameter, and betweenness centrality. The betweenness centrality of each correlated genus was calculated to evaluate its importance in the microbial network [40].

3. Results

3.1. Edaphic Properties

Unconstrained PCoA analysis showed that the soil properties for each of the three treatments generally clustered into distinct groups. The first two axes were separated based on cultivation system, and soil depth; these components explained 68.03% and 18.38% of variation in soil properties, respectively (Figure 1a). PERMANOVA further confirmed that both soil depth and exposure to 7 years of each cultivation system had significant impacts on the soil properties ($p = 0.019$ and 0.001 , respectively, Table S1).

For the depth effect, we found that water content, pH, and the C/N ratio were significantly lower at 0–10 cm than 10–20 cm, whereas EC, bulk density, NO_3^- -N, urease activity, and total C and N generally showed the opposite trend in the same system ($p < 0.05$, Table 2). In terms of management, soil pH was significantly higher in NF (6.92 and 7.05 at 0–10 cm and 10–20 cm soil depth, respectively) than CF and CP ($p < 0.05$, Table 2); similar trends were observed for water content and nitrate reductase activity at both soil depths. At a soil depth of 0–10 cm, total C, total N, and urease activity were significantly higher in NF than CP and CF. However, the lowest EC, bulk density, and NO_3^- -N values were observed in NF at 0–10 cm. Notably, soil bulk density (1.1 g cm^{-3}) was significantly higher in CF than the other treatments. The trends in EC, bulk density, NO_3^- -N values, and nitrate reductase activity at 10–20 cm were similar to those in the 0–10 cm layer. Briefly, NF had higher soil quality (e.g., bulk density, EC, and total C) and enzyme activities (i.e., urease activity and nitrate reductase activity) compared to the other systems.

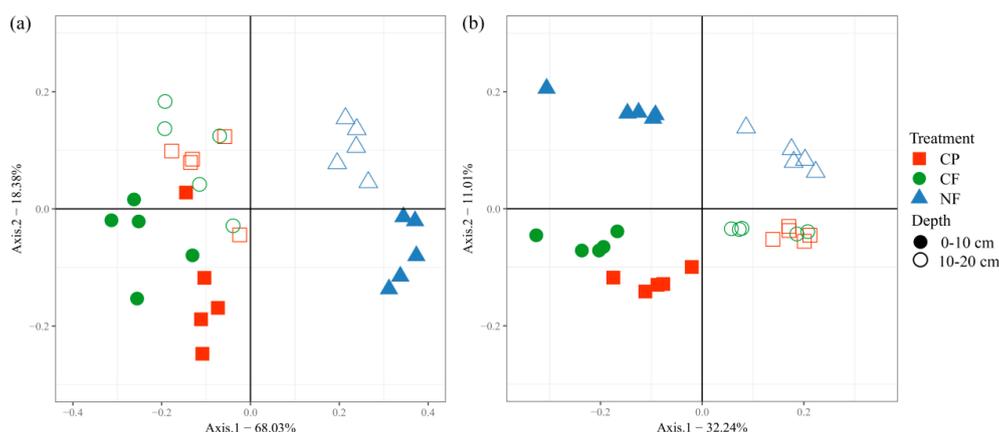


Figure 1. Unconstrained principal coordinate analysis (PCoA) based on Euclidean distances and the Bray–Curtis distance, respectively, for the effect of soil depth and agricultural practice on (a) soil properties and (b) microbial community structure. CP, conventional practice with chemicals; CF, conventional practice without chemicals; NF, natural farming.

Table 2. Soil physical, chemical, and biological properties for the three cultivation systems at two soil depths.

Depth	0–10 cm			10–20 cm		
	CP	CF	NF	CP	CF	NF
Water content (%)	34.38 ± 0.18 e	33.55 ± 0.17 f	37.87 ± 0.33 a	35.43 ± 0.16 c	34.50 ± 0.15 d	36.68 ± 0.15 b
Bulk density (g cm ⁻³)	1.01 ± 0.03 b	1.10 ± 0.03 a	0.96 ± 0.02 bc	0.93 ± 0.01 cd	0.96 ± 0.02 bc	0.87 ± 0.03 d
pH	6.00 ± 0.04 e	5.94 ± 0.06 e	6.92 ± 0.03 b	6.38 ± 0.01 d	6.50 ± 0.02 c	7.05 ± 0.01 a
EC (dSm ⁻¹)	0.14 ± 0.03 a	0.10 ± 0.01 ab	0.08 ± 0.01 bc	0.09 ± 0.00 bc	0.08 ± 0.00 bc	0.05 ± 0.00 c
NH ₄ ⁺ -N (mg kg ⁻¹)	6.70 ± 1.15 ab	5.56 ± 1.73 ab	8.78 ± 3.81 a	1.52 ± 0.53 b	2.54 ± 0.78 b	3.72 ± 0.87 ab
NO ₃ ⁻ -N (mg kg ⁻¹)	33.24 ± 10.39 a	31.90 ± 5.92 a	12.78 ± 1.69 b	16.50 ± 3.19 b	12.32 ± 0.91 b	8.80 ± 0.80 b
Urease activity ¹	45.27 ± 8.26 b	22.27 ± 5.71 c	69.05 ± 7.96 a	18.26 ± 3.37 c	17.01 ± 4.54 c	21.48 ± 2.56 c
Nitrate reductase activity ²	9.79 ± 1.84 c	3.18 ± 0.40 c	101.92 ± 6.35 a	9.21 ± 2.15 c	7.09 ± 1.37 c	49.26 ± 3.48 b
TC (%)	4.21 ± 0.05 c	4.55 ± 0.02 b	4.94 ± 0.11 a	4.00 ± 0.09 d	4.48 ± 0.07 b	4.25 ± 0.03 c
TN (%)	0.38 ± 0.01 b	0.39 ± 0.01 b	0.43 ± 0.01 a	0.33 ± 0.01 d	0.35 ± 0.00 c	0.34 ± 0.01 cd
C/N	10.99 ± 0.25 b	11.55 ± 0.15 b	11.38 ± 0.08 b	12.27 ± 0.20 a	12.73 ± 0.22 a	12.37 ± 0.15 a

Note: Means ± standard error values followed by different letters differ significantly within the same row; Duncan's multiple range test ($p < 0.05$, $n = 5$). CP, conventional practice with chemicals; CF, conventional practice without chemicals; NF, natural farming practice; EC, electrical conductivity; TC, total carbon; TN, total nitrogen; C/N, ratio of carbon and nitrogen. ¹: mg kg⁻¹ soil-37 °C 2 h. ²: mg kg⁻¹ soil-25 °C 24 h.

3.2. Microbial Community Structure

A total of 985,542 16S rRNA sequences were obtained by pyrosequencing from the 30 samples (three cultivation systems and two depths; five replicates each); the sequences clustered into 3979 to 5357 OTUs per sample with 97% similarity according to the Greengenes database (Table 3). Rarefaction curves increased steadily as the number of sequences increased (Figure S2), while the Good's coverage of all samples ranged from 0.95 to 0.97 (Table 3), indicating that the sample size was sufficient for subsequent data analysis.

Unconstrained PCoA indicated that microbial β -diversity clearly clustered based on soil depth and cultivation system; these components explained 32.24% and 11.01% of variation in microbial β -diversity, respectively (Figure 1b). PERMANOVA revealed that soil depth ($p < 0.001$) and cultivation system ($p = 0.047$) led to significant shifts in overall microbial community composition (Table S1). Univariate analysis showed that microbial abundance (i.e., the ACE and Chao 1 indices) and diversity (i.e., the Shannon index) were significantly higher ($p < 0.05$) in the 0–10 cm than 10–20 cm soil layer, except for the CP plot (Table 3). At 0–10 cm, microbial richness was higher in CF and NF than CP, while microbial diversity was significantly higher in CF than CP (Table 3). However, there was no difference in microbial richness or diversity between any cultivation systems at 10–20 cm.

Table 3. Richness estimators and diversity index for different soil depths and cultivation systems at 97% identity threshold.

Depth	Cultivation System	Reads	OTUs	Coverage (%)	ACE	Chao1	Shannon
0–10 cm	CP	35283	4680	0.97	1229 ± 89 c	1140 ± 75 c	5.62 ± 0.32 bc
	CF	32882	5357	0.95	1756 ± 137 a	1675 ± 175 a	6.91 ± 0.98 a
	NF	31859	4969	0.95	1578 ± 131 ab	1475 ± 100 ab	6.39 ± 1.20 ab
10–20 cm	CP	32966	3986	0.97	1197 ± 239 c	1082 ± 207 c	5.30 ± 0.29 c
	CF	31229	4232	0.96	1364 ± 330 bc	1238 ± 292 bc	5.41 ± 0.53 c
	NF	32889	3979	0.97	1245 ± 271 c	1127 ± 228 c	5.34 ± 0.25 c

Note: Different letters indicate statistically significant differences according to Duncan's test ($p < 0.05$; $n = 5$). CP, conventional practice with chemicals; CF, conventional practice without chemicals; NF, natural farming practice; OTUs, operational taxonomic units; ACE, abundance-based coverage estimator.

To clarify microbial community composition within the three cultivation systems, the phylum-level dataset was analyzed first; pyrosequencing generated 38 bacterial phyla and two archaea phyla across all soil samples (Figure 2). The most dominant phylum across all treatments was Firmicutes (53.56%–79.12%). The relative abundance of this phylum was more abundant in CP (68.47%) and decreased in the order of NF > CF at 0–10 cm soil depth. Furthermore, the relative abundance of Firmicutes was higher at a soil depth of 10–20 cm (76.28%–79.13%) than 0–10 cm (53.56%–68.47%).

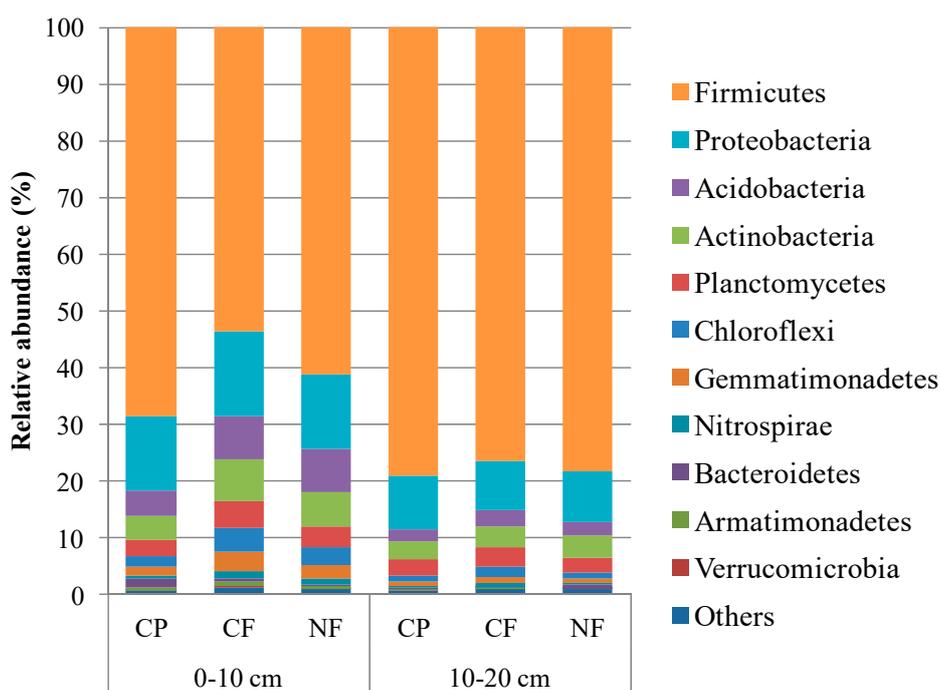


Figure 2. Relative abundance of dominant microbial phyla in the three cultivation systems at different soil depths. CP, conventional practice with chemicals; CF, conventional practice without chemicals; NF, natural farming practice.

LEfSe analysis (LDA score > 3.0) was applied to identify microbes specifically enriched at the phylum and genus levels in the different cultivation systems at both soil depths. CF had the highest numbers of differential taxa of any cultivation system at 0–10 cm (Figure 3). The relative abundances of the OTUs from the phyla Planctomycetes, Acidobacteria, and Nitrospirae were significantly higher in CF. At the genus level, *Gemmata* (Planctomycetes) was the most differential taxon in CF (LDA score = 3.75), followed by *Planctomyces* (Planctomycetes), *Terracoccus* (Actinobacteria), *Pseudonocardia* (Actinobacteria), *Candidatus Koribacter* (Acidobacteria), *Devosia* (Proteobacteria), *Nitrospira* (Nitrospirae), *Methylobacterium* (Proteobacteria), and *Pirellula* (Planctomycetes). In addition, the relative abundances of the phylum

Bacteroidetes and genera *Erwinia* (Proteobacteria), *Sphingobacterium* (Bacteroidetes), *Comamonas* (Proteobacteria), *Enterobacter* (Proteobacteria), *Brenneria* (Proteobacteria), and *Chryseobacterium* (Bacteroidetes) were significantly enriched in CP. Notably, the preferential taxa in NF were the genera *Pedomicrobium* (Proteobacteria), *Solirubrobacter* (Actinobacteria), and *Lentzea* (Actinobacteria). It is worth noting that no distinct taxonomic differences (LDA > 3.0) were observed between the three cultivation systems in the 10–20 cm soil layer.

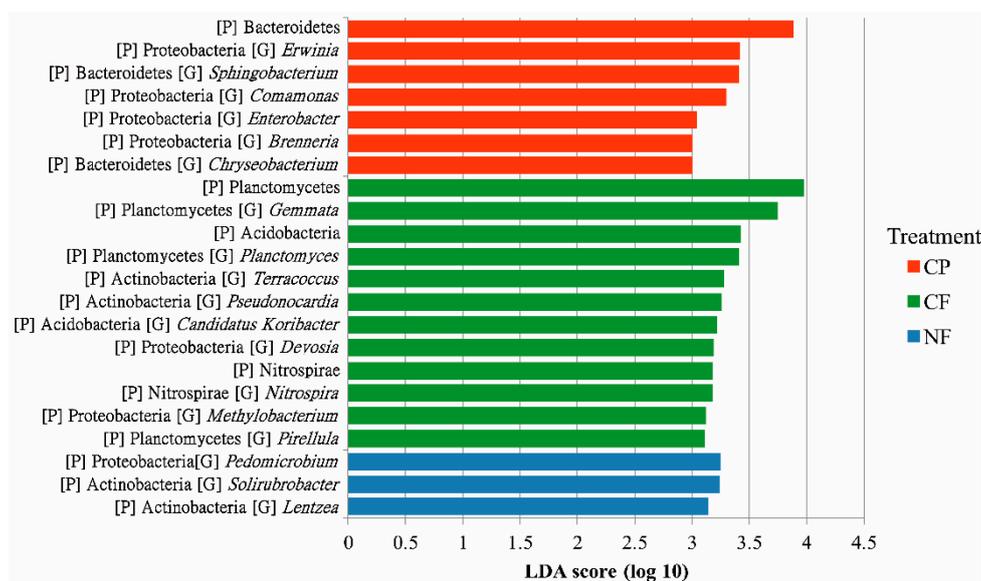


Figure 3. Linear discriminate analysis (LDA) of effect size (LEfSe) to identify preferential taxa at the phylum [P] and genus [G] levels in each agricultural practice at a soil depth of 0–10 cm. Only taxa with an LDA score > 3.0 are shown. No taxonomic differences were observed between the three agricultural practices at a depth of 10–20 cm. CP: conventional practice with chemicals; CF: conventional practice without agrochemicals; NF: natural farming.

3.3. Microbial Network Analysis

The fifty most abundant genera across all treatments were analyzed using Spearman's rank correlation analysis. Strong (absolute value of $r > 0.6$), significant ($p < 0.01$) correlations were used to construct clustering microbial networks for the 0–10 cm and 10–20 cm soil layers (Figure 4). In terms of the topological properties of the microbial networks, the node number, interaction (edge) number, and network diameter were 48, 239, and 7 in the 0–10 cm layer and 39, 64, and 9 in the 10–20 cm layer, respectively, suggesting a more interconnected network in the topsoil than deeper soil. Proteobacteria was the most abundant phylum in both networks, accounting for 42% and 31% of total nodes at 0–10 cm and 10–20 cm, respectively. The two genera with the highest betweenness centrality were *Flavisolibacter* and *Candidatus Solibacter* at 0–10 cm and *Candidatus Nitrososphaera* and *Leuconostoc* at 10–20 cm, these genera may play important roles to maintain the network structure at the respective soil depths.

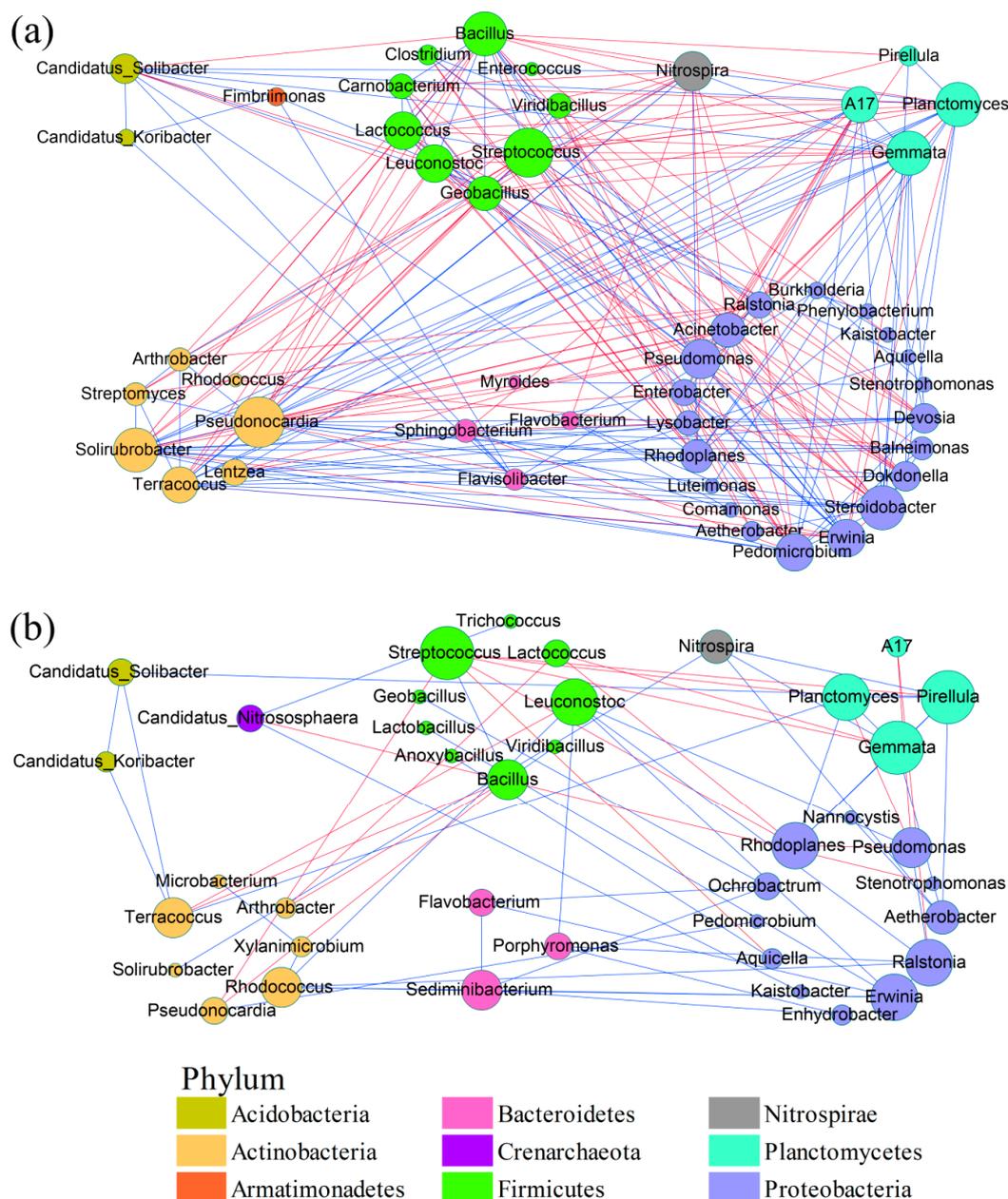


Figure 4. Overview of microbial network associations in response to different cultivation systems at soil depths of (a) 0–10 cm and (b) 10–20 cm. Correlations between the fifty most abundant genera in each soil layer were selected for each network. Blue and red edges represent significant, strongly positive ($r > 0.6$, $p < 0.01$) or negative ($r < -0.6$, $p < 0.01$) correlations between two nodes. The size of each node is proportional to the number of edges associated with the node.

4. Discussion

In this study, we aimed to explore the impacts of different agricultural managements and soil depth on soil properties and microbial community composition under long-term field conditions. Although the experimental site is relatively small (7.2 m × 22.8 m) compared to other field experiments [8–11], soil chemical analysis has been conducted every two or three years since the experimental field was established and showed that the beneficial effects of natural farming on soil properties improved over time (Figure S2). In addition, our findings are consistent with several studies showing that organic farming (e.g., natural farming) improves soil properties compared to conventional farming [1,5,21]. These results imply that the experimental field is well-maintained and provides reliable samples

for further pyrosequencing analysis. We observed several differential taxa under each treatment, which may provide ecological information on how soil microbes respond to different agricultural managements (Figure 3). Furthermore, network analysis showed integrated microbial interactions at both soil depths. The key genera with the highest betweenness centrality might play fundamental roles in maintaining soil microbial network structure (Figure 4).

4.1. Edaphic Properties Vary in Response to Agricultural Management and at Different Soil Depths

It is well known that organic farming improves the soil environment compared to conventional farming; this effect is mainly attributed to the relatively sustainable agricultural practices in organic farming, such as organic material amendment [12], mulching with plant residue [21], crop rotation [22], and reduced tillage [25]. Similarly, our results showed that natural farming led to better soil quality than other cultivation systems; these improvements may be explained by the integrated agricultural practices in NF. For example, soil pH was significantly higher in NF than in the treatments with chemical fertilization (Table 2). This finding is in line with a recent long-term fertilization field study, which indicated that chemical fertilization increased soil acidity through acidification and nitrification [24]. Furthermore, NF soil had a higher moisture content and total C and lower bulk density than soil from other cultivation systems. These changes are strongly related to organic fertilization [12], straw mulching [21], and reduced tillage [25] in the NF plot, which have been shown to maintain water content and improve soil structure by increasing the carbon content in the soil. Previous studies also indicated that an improvement in the organic C content of soil could lead to increased microbial activity and/or enzyme activities (e.g., urease) [11,15]. A similar result was observed in the NF plot, which had the highest urease and nitrate reductase activities compared to the other cultivation systems. This finding suggests that NF improves microbial N transformation in the soil by providing sufficient available C sources [41]. Notably, we found that urease activity was significantly higher in CP than CF. One possible explanation for this observation is the stimulation of urease activity by application of pesticides [42]. Together, our findings indicate that NF improves the soil ecosystem, while conventional farming methods—specifically the application of mineral fertilizer—negatively affect soil properties over the long-term.

In the 0–20 cm soil layer, the soil pH was lower in the topsoil than the deeper soil. This could be explained by the acceleration of acidification and nitrification processes induced by fertilization [24]. In addition, higher levels of nutrients (e.g., EC, $\text{NH}_4^+\text{-N}$, and $\text{NO}_3^-\text{-N}$, total C and N) and enzyme activities (e.g., urease and nitrate reductase activity) were observed in the top soil compared to the deeper soil layer within each cultivation system. These results are in line with previous studies, which found that nutrient levels and microbial biomass decreased as soil depth increases [24]. Indeed, most fertilizers, root exudates, and plant debris accumulate in the top soil, and thus further stimulate microbial growth and activity [41]. Similarly, we observed significantly higher microbial abundance and diversity at 0–10 cm than 10–20 cm (Table 3).

4.2. Characteristics of the Soil Microbial Community under Different Agricultural Managements

Soil microbial abundance and diversity are fundamental to the stability of soil ecosystems [5,11]. Our results revealed lower microbial richness and diversity in CP than CF. The only difference in agricultural practices between CP and CF is the use of chemicals (e.g., pesticides). This observation indicates that application of chemicals negatively affects microbial reproduction, in line with previous studies [5,17]. Similarly, NF led to significantly higher microbial abundance and diversity than CP. This finding is consistent with previous studies showing that organic management increased microbial alpha diversity in different crop production systems (i.e., vegetable, tea, and rice), and these improvements were mainly related to avoidance of agrochemicals and application of organic fertilizer [5,11]. However, there was no difference in microbial richness and diversity between CF and NF. This might be due to the CF agricultural practices, including mineral fertilization and conventional tillage. Both practices have been reported to increase soil microbial size [15] and diversity [43] by

increasing the availability of easily assessable nutrients and stimulating degradation of soil organic matter. Therefore, we propose that application of chemicals (e.g., pesticides) may be the main factor that alters soil microbial alpha diversity while other agricultural practices (e.g., tillage, straw mulching, and fertilization) have smaller effects on soil microbial alpha diversity at our experimental site.

4.3. Agricultural Management-Associated Microbial Taxa

LEfSe analysis (LDA > 3.0) revealed preferential taxa in each cultivation system in only the 0–10 cm soil layer, not at 10–20 cm, indicating the effects of agricultural management on the soil microbial community are more substantial in topsoil than the deeper soil [20]. In turn, the topsoil (0–10 cm) was the optimal sampling depth to explore the emergence of differential taxa in response to different agricultural practices in the present study.

Given that soil microbes sensitively respond to changes in the soil environment, exploring distinct microbial taxa under different agricultural managements may reveal the ecological importance of the predominant taxa in each cultivation system. Bacteroidetes was the most significantly enriched bacterial phylum in CP. OTUs belonging to this phylum have previously been classified as copiotrophic bacteria and are involved in C mineralization [44]. At the genus level, we observed OTUs assigned to the genera *Sphingobacterium* and *Chryseobacterium* of this phylum. *Sphingobacterium* spp. and *Chryseobacterium* spp. have the ability to decompose xenobiotics (e.g., pesticides and carbazole) [45,46]; therefore, enrichment of these genera in the CP plot may be due to the use of agrochemicals [47]. Similarly, OTUs within the genus *Comamonas* have been frequently associated with degradation of complex compounds [13], and were strongly associated with CP. In addition, in CP, we also observed abundant OTUs belonging to the genera *Erwinia*, *Brenneria* and *Enterobacter*, which are known to be plant pathogenic microorganisms. *Erwinia* spp. are known as a soft rot pathogen of Chinese cabbage [16], while *Brenneria* spp. have been reported to induce drippy pod in white lupin [48]. *Enterobacter* spp. belonging to the family Enterobacteriaceae have been shown to possess pathogenic genes related to human, animal, and plant hosts [49]. Indeed, the increased abundance of pathogenic taxa may be attributed to lower resistance to pathogen invasion, as indicated by the lowest microbial abundance and diversity of the CP plot (Table 3) [50].

Long-term chemical fertilization stimulates recalcitrant C degraders in soils due to the reduction in the labile C content associated with the limited input of organic materials [51]. Consistently, we observed a range of preferential OTUs in the CF plot involved in C degradation. For example, OTUs belonging to the phylum Planctomycetes are recognized as important degraders of complex C [52]. In particular, we further observed differential OTUs belonging to three genera within this phylum: *Gemmata*, *Planctomyces*, and *Pirellula*, which are known to degrade soil organic materials [53,54]. Similarly, OTUs that were abundant in CF are also involved in utilization or metabolism of organic substances, including Actinobacteria (*Pseudonocardia* and *Candidatus Koribacter*) [55,56] and Proteobacteria (*Devosia* and *Methylobacterium*) [57,58]. Since the fertilization regime was the same between CP and CF, these distinct taxa may be sensitive to the toxic agrochemicals used in the CP plot. Although most studies indicate that pesticides have no effect on soil C utilization [47], our findings imply that exogenous pesticides highly disturb the composition of C degraders in the presence of mineral fertilizer application. Additionally, the abundance of OTUs affiliated to the phylum Nitrospirae and its genus *Nitrospira* were significantly increased in CF; these are well-known nitrifying bacteria. Previous studies reported that Nitrospirae were highly associated with chemical fertilizer-amended soil in a 32-year field trial [24]. Therefore, enrichment of Nitrospirae in CF may be related to the high NO₃⁻-N content of CF soil and high substrate affinity of these nitrifying bacteria.

OTUs from the genera *Pedomicrobium*, *Solirubrobacter* and *Lentzea* were highly associated with NF. *Pedomicrobium* spp., chemoorganotrophs that generally utilize C substrates [12], were abundant in the soil of a sustainable agricultural system in Mexico [59]. *Solirubrobacter* spp. prefer soil with more stable aggregates [60] and were present at higher abundance in organically managed soil than conventionally managed soil [10]. These findings suggest that the enrichment of *Solirubrobacter* spp. in organic or

natural farming systems may have implications for sustainable agricultural systems. In addition, *Lentzea* spp. have been reported as plant growth-promoting bacteria [61]. Together these results indicate specific taxa may prefer different agricultural systems, and generate concern that continuous use of agrochemicals stimulates the growth of plant pathogens and xenobiotic-degrading species in the soil. In contrast, NF not only improved soil quality, but also stimulated beneficial microbial linkages, which may in turn help to further enhance the sustainability of this cultivation system.

4.4. Robustness of and Key Genera in Microbial Communities

Based on the mathematical interaction, network analysis of the dominant microbial communities in each cultivation system showed a higher level of complexity (in terms of the number of nodes and edges) in the 0–10 cm soil layer than the 10–20 cm layer (Figure 4, which may be considered as a sign of stronger resilience to exogenous microbes in topsoil [62]. This may be due to the fact agricultural practices mainly affect topsoil, with fewer disturbances to the deeper soil layers. Additionally, the two genera with the highest betweenness centrality in the 0–10 cm soil layer, *Flavisolibacter* (Bacteroidetes), and *Candidatus Solibacter* (Acidobacteria), may play fundamental roles in the microbial network of the topsoil. Recent stable isotope probing experiments revealed *Flavisolibacter* spp. are a major group involved in plant C utilization, and are highly associated with phosphorus turnover and other bacterial species [14]. *Candidatus Solibacter* spp. are slow-growing chemoorganotrophs that play a key role in the C cycle in terrestrial soil [63,64]. Furthermore, a recent study reported that *Candidatus Solibacter* spp. exhibited the highest positive correlations with other bacteria in an organic-conventional soil bacterial network [8]. In the 10–20 cm layer, the two genera with the highest betweenness centrality were *Candidatus Nitrososphaera* and *Leuconostoc*. *Candidatus Nitrososphaera* spp. are important ammonia-oxidizer archaea that are ubiquitous in soil [65]. *Leuconostoc* spp. act as antagonists to inhibit the growth of soil-borne pathogens (e.g., *Rhizoctonia solani* and *Pythium aphanidermatum*) [66]. Indeed, the possibility of such exogenous pathogens replacing the key genera and resulting in destruction of the soil microbial network is a concern [67]. Overall, our results demonstrate stronger microbial interactions in topsoil than deeper soil. The resulting key genera may play crucial roles in the soil ecosystem and the ecological importance of these genera to the maintenance of soil microbial networks may provide a new direction of research. However, the specific ecological mechanisms governing microbial networks require further investigation.

5. Conclusions

This study demonstrates both soil depth and 7 years' implementation of different agricultural management practices significantly influence soil properties and microbial community structure. The topsoil (0–10 cm) experiences greater disturbances and exhibited more stable and more complex microbial networks in response to different agricultural practices than the deeper soil (10–20 cm). In addition, differential taxa were only observed in the topsoil, suggesting 0–10 cm is the optimal sampling depth for exploring agricultural management-associated microbial groups. NF improved soil physical, chemical, and biological properties and led to a distinct microbial community structure compared to soils under conventional management. The differential taxa in NF, such as *Pedomicrobium* and *Solirubrobacter*, have previously been related to the improved soil conditions in originally managed or sustainable agroecosystems. In contrast, CP enriched the abundance of a range of genera related to plant pathogens (e.g., *Erwinia* spp. and *Brenneria* spp.) and xenobiotic degraders (e.g., *Sphingobacterium* spp. and *Chryseobacterium* spp.). Taken together, this study indicates NF beneficially alters the soil environment and ecosystem and could be regarded as an appropriate management for sustainable agricultural production.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2071-1050/11/11/3131/s1>, Figure S1: Dynamic changes of (a) pH, (b) EC, (c) total C and (d) total N in the 0–10 cm soil depth managed by CP, CF and NF since 2009. Figure S2: Rarefaction curves for the soil samples at (a) 0–10 cm and (b) 10–20 cm based on

observed species at the 97% identity threshold, Table S1: PERMANOVA of the significance of the effect of soil depth and cultivation system on soil properties and microbial community structure.

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