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Changes on bacterial and fungal soil communities in long-term organic cropping systems

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Abstract: Microbial communities play a key role in sustainable agriculture. However, we still need more information, to understand the complex response of the microbial community to long-term organic farming, which aims to reduce synthetic fertilizer and pesticide use in order to produce sustainably and improve soil quality. We have assessed the long-term effect of two organic cropping systems and a conventional system on the microbial soil community structure using high-throughput sequencing analysis. We analyzed the link between these communities and changes in soil properties and crop yield. Results showed that the crop yield was similar among the three cropping systems. Soil properties, such as total organic carbon, nitrogen, ammonium, magnesium and boron, influenced changes in the bacterial community structure. A linear discriminant analysis effect size (LEfSe) showed different bacteria and fungi as key microorganism of each of the three different cropping systems, in addition, our results reflected that fungal community were more sensitive than bacteria to cropping system. This research provides an insight about changes occurred in soils, especially in microbial communities considering the effect of that changes in crop yield which were remained stable among the different cropping systems.

Keywords: compost; high-throughput sequencing; sheep manure; soil properties; crop yield

1. Introduction

Currently, one third of agricultural soils worldwide are moderately or highly degraded, affecting production [1]. Chemical fertilizers, pesticides and herbicides have been commonly used to maintain soil fertility and crop production in conventional farming [2], and this has generated an increase in greenhouse gas emissions and soil degradation and a decrease in soil biodiversity [3,4]. In this context, organic farming is of growing interest. Organic farming includes the use of organic fertilizers, such as compost, manure, green manure and bone mean, and places emphasis on techniques like rotation with companion plants or intercropping and pest and disease control by natural methods, avoiding systhetic chemical compounds [5]. Some studies have reportes that organic crop production is between 10-30% lower than in conventional farming, with differences

depending on the crop species, growing conditions and management practices [6]. Nonetheless, in recent years, new studies have highlighted that long-term organic management can contribute to crop yields similar to those found in conventional farming, once the system has been stabilized after initial years with a remarkable reduction in crop yields [7,8]. This change is mostly related to higher soil quality and microbial diversity and activity [8]. Conflicting results show however that little is known about the relative variability of organic systems compared to conventional ones. In the European Union, almost 180 million hectares are dedicated to agriculture, of which around 13 million were dedicated to organic farming in 2018. Spain is the EU country with the largest area devoted to organic farming [9], with more than 2.2 million hectares accounting for 16.7% of the total farmed land in the country.

Soil microorganisms play an important role in ecosystem processes such as carbon cycling, decomposition, nutrient cycling, and soil aggregate formation [10], although determining this relationship is very complex [11]. Understanding how these microorganisms respond to organic matter, inorganic fertilizers and soil management can thus be indicative of soil health for crop production [12,13]. Some studies have reported that after long-term organic farming, microbial diversity, soil sustainability and beneficial microorganisms involved in plant health were higher than in conventional farming [14–16]. However, contrary results have also been found. E.g., Bell et al. [17] and Krishnaraj and Sabale [18] found that organic cropping systems showed no significant differences, or even lower microbial diversity, compared to conventional cropping. It is therefore of great interest to explore how soil microbial communities change across different farming types. Approaches like the high-throughput analysis of bacterial and fungal communities can show taxonomic shifts shaping patterns of ecological interactions that regulate the structure, function, and resilience of soil microbial communities under organic farming compared to conventional farming.

The aim of this paper was to investigate the effect of three different long-term cropping systems: a conventional system (Conv) and two organic systems [organic cultivation with manure compost and compost Tea (Org_C) and organic cultivation with manure (Org_M)] on soil bacterial and fungal communities, studied through high-throughput sequencing analysis focusing on relationship between these communities and physico-chemical soil properties and crop production. We hypothesized that a) organic systems would not have significant differences on crop production to the conventional system, since once an organic system is stabilized, soil functionality is improved, contributing to high nutrient availability and soil health; b) both organic systems (Org_C and Org_M) would promote changes on microbial structure and abundance compared with conventional system and c) that changes promoted by cropping system can be either beneficial or detrimental to plants, thus influencing soil stability and quality.

2. Materials and Methods

2.1. Experiment description and sampling

The study site was located in the Campo de Cartagena, an agrarian region of southeastern Spain. The soil was a Haplic Calcisol (Loamic, Hypercalcic) IUSS [19]. The area has a mean annual temperature of 17.5ºC, a mean annual precipitation of 280 mm and an annual potential evapotranspiration of 1300 mm. The site has been under vegetable cultivation since the early 1990s, using drip fertigation, rotation and multiple cropping. Three cropping systems were selected for this study: (1) a conventional system using a yearly addition of sheep manure as organic amendment, inorganic fertilizer for fertigation and pesticides (Conv); (2) an organic system using a yearly addition of compost, amino acids to provide N and compost tea to provide organic compounds and nutrients for fertigation, no pesticides and cover crops of *Avena sativa* and *Vicia sativa* between cropping seasons (Org_C); (3) an organic system using a yearly addition of sheep manure, amino acids as fertigation to provide N and no pesticides (Org_M). The three cropping systems are described in detail in Table 1.

Table 1. Management characteristics of the three cropping systems.

Cultivation system	Conv	Org_C	Org_M
Geographical coordinates	37° 48' 18.5" N; 0° 51' 49.2" W	37° 51' 39.3" N, 0° 54' 03.3" W	37° 49' 30.2" N, 0° 52' 28.4" W
Crop 2017-2018 season	*Brassica oleracea var. sabellica*		
Harvest	Manual on 20-25 February 2018. Crop residues were incorporated in the soil		
Crops grown in previous years	*Apium graveolens / Cucumils melo* (2016/2017) *Lactuca sativa / Brassica oleracea var. Italica* (2015/2016) *Apium graveolens / Cucumils melo* (2014/2015) *Brassica oleracea var. Italica / Capsicum annum* (2013/2014) *Foeniculum vulgare/ Cucurbita moschata* (2012/2013)		
Organic amendments (amount per year)	15,000 kg ha^{-1} sheep manure;	10,000 kg ha^{-1} sheep compost; compost tea*	15,000 kg ha^{-1} sheep
Fertilizers (amount per year)	15 kg ha^{-1} ENTEC solub 21 (ammonium sulfate with inhibition of nitrification); 10 L ha^{-1} phosphoric acid; 15 kg ha^{-1} calcium nitrate; 10L ha^{-1} nitric acid	10 L ha-1 EcoZen NPK 2-2-7 (aminoacids); 10 L ha-1 Sunfol veg agri 12% (aminoacids)	
Pesticides	Linuron; Indoxacarb 30%; Cypermethrin;Lambda cihalotrin 10%; Imidacloprid 20%; Spinosad; Azadirachtin 3.2%; Emamectin 0.85%; Clortalonil 50%; Difenoconazol 25%; Azoxystrobin 2.5%; Propamocarb 52%; Ciflufenamid	No application of chemical pesticides	

Conv, Conventional system; Org_C, Organic cultivation with sheep manure compost and compost tea; Org_M, Organic cultivation with sheep manure. *The compost tea was made on each farm by steeping mature compost in water for 24h.

The characteristics of the sheep manure, compost and tea compost are shown in Table S1. The amount of compost added annually in the Org_C treatment before each crop cycle was lower than the amount of manure added before each cycle in the Conv or Org_M treatments; because soluble organic compounds were continuously added during the crop cycle by fertigation with the compost tea in Org_C, the organic matter was not added all at once but rather over an extended period of time (Table 1).

Five different plots (~1 ha) were selected for each cropping system. One composite sample derived from ten subsamples (0-10 cm depth) was collected with an auger from

each plot in February 2018 after the harvest of a leaf cabbage crop (*Brassica olearacea* var. sabellica).

Soil samples were separated into two aliquots. One was kept at ambient temperature for physicochemical analyses and the other was stored in a cool box with ice for biological analysis. Samples were taken to the lab immediately. The soil was air-dried for one week for physicochemical analyses and sieved at < 2mm. The soil for biological analysis was sieved at < 2mm and stored at -20ºC. Soil cores were taken using steel cylinders to determine soil bulk density [20].

2.2. Soil properties and crop yield

Actual field soil moisture content (FMa) was gravimetrically determined according to De Angelis [21]. The cation exchange capacity (CEC) and exchangeable Ca^{2+}, Mg^{2+}, K^+, Na^+ were determined using $BaCl_2$ as exchangeable cation following the ISO international standard method 13536, ISO [22]. The soil water content at wilting point (SWW) and soil water content at field capacity (SWFC) were calculated by the retention curve method [23], in which moist samples were dried by raising the air pressure in an extractor with a porous ceramic plate [24]. The soil pH and electrical conductivity (EC) were measured in deionized water (1:5 w/v). The total organic carbon (TOC), inorganic carbon (IC) and total nitrogen (TN) were determined by an elemental CHNS-O analyzer (EA-1108, Carlo Erba); the $CaCO_3$ content was calculated from IC. The particulate organic carbon (POC), defined as a fresh or decomposing organic material, was measured according to Cambardella and Elliot [25]; in long-term experiments, POC can be used as an early indicator of soil organic matter (SOM), corresponding to the functional pool of organic matter stabilized by specific mechanisms [26]. Soil NH_4^+ was extracted with 2M KCl in a 1:10 soil:extractant ratio and calorimetrically measured [27,28]. Soil NO_3^- was extracted with deionized water in a 1:10 soil:extractant ratio and measured by ion chromatography (Metrohm 861). Available P (P) was measured using the Olsen method [29]. Available Fe, (Fe) available Mn (Mn), available Cu (Cu) and available Zn (Zn) were extracted by chelation using DTPA (1:2 w/v) [30,31]. Available B (B) was extracted with deionized water (1:5 w/v) at 50ºC [32]. Available nutrients were measured using ICP-MS (Agilent 7500CE). Total pesticides were determined by the QuEChERS method [33], according to which, 5 g of a homogenized sample was extracted with 10mL acetonitrile containing 1% acetic acid. Pesticides were analyzed through Liquid Chromatography Triple Quadrupole Mass Spectrometry (LC-MS/MS; TQS MS linked to a Waters Acquity UPLC system) and Gas Chromatography Triple Quadrupole Mass Spectrometry (GC-MS/MS; Agilent 7890B GC coupled to an Agilent 7010B MS system).

The leaf cabbage yield (kg ha-1) was calculated based on the weight of all marketable plants (suitable for sale according to size) in each plot.

2.3. DNA extraction, PCR and sequencing

Soil DNA was extracted from 1 g of soil (wet weight) using the DNeasy Power Soil Kit (Qiagen). The quantity and quality of DNA extracts were quantified using a Qubit 3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, USA) and a NanoDrop 2000 fluorospectrometer (Thermo Fisher Scientific, Waltham, MA, USA).

The bacterial community was determined through the Next-Generation-Sequencing of bacterial 16S hypervariable regions using the Ion Torrent™ Personal Genome Machine™ (PGM) System. Bacterial 16S regions were amplified using an Ion 16S™ Metagenomics Kit (Thermo Fisher Scientific) with two different degenerate primer sets to amplify regions V2-4-8 and V3-6, V7-9. Amplified 16S amplicons were then processed using an Ion Xpress™ Plus Fragment Library Kit in combination with an Ion Xpress™ Barcode Adapter 1-96 Kit (Thermo Fisher Scientific). All purification processes between incubation and the amplification reactions of library preparation were processed using DynaMag™-2 magnetic racks (Thermo Fisher Scientific) and an AMPure XP Purification Kit (Beckman Coulter). Library preparation and barcoding were followed by determina-

tion of size and concentration of the final libraries using an Agilent 2100 Bioanalyzer system and the Agilent High Sensitivity DNA kit. Sequencing templates were prepared using an Ion One Touch 2 System and an Ion PGM™ Hi-Q™ View OT2 Kit (Thermo Fisher Scientific). The sequencing reaction was performed using Ion Torrent PGM with an Ion PGMTM Hi-QTM View Sequencing Kit (Thermo Fisher Scientific).

Fungal libraries were prepared using a protocol based on the method proposed by Smith and Peay [34] with some modifications. Sequencing libraries were produced by PCR amplification using the primer pairs ITS1f-ITS2 tailed with the Illumina adapters. The reverse primers were barcoded using the 12-base Golay barcodes [35]. DNA extraction had a minimum concentration of 10ng/µl with a least 200ng provided. The PCR amplifications were conducted in a final volume of 30 µl containing 3 µl of buffer 10X, 0.7 µl of each primer (10 mM), 0.9 µl of 50 mM MgSO4, 0.6 µl of 10mM dNTP, 2 µl of template DNA (10ng/mL), 21,98 µl of PCR-grade water and 0.12 µl of Invitrogen Platinum Taq DNA polymerase High Fidelity (Cat N° 11304-011) using the following conditions: 3 min initial denaturation at 95 ºC, followed by 35 cycles of denaturation at 95 ºC for 45 s, annealing at 50 ºC for 1 min, extension at 72 ºC for 1 min, and a final extension at 72 ºC for 10 min. PCR products were cleaned up from primers using Agencourt AMPure XP beads (Beckman Coulter) following the manufacturer's instructions. The PCR products were checked on a Bioanalyzer DNA 1000 kit to verify the size. Amplicons were quantified with Qubit using the dsDNA HS Assay kit. Amplicon libraries were sequenced on the Illumina MiSeq machine together with a 10% PhiX control library to generate 300 bp paired end reads.

2.4. Sequencing data processing

For bacterial raw sequences, barcodes and primers were trimmed according to the BaseCaller application. Sequences were denoised with ACACIA [36], and low quality sequences were discarded using the Quantitative Insights into Microbial Ecology (QIIME) pipeline [37] from the Microbiome Helper Virtual Box [38]. Briefly, bacterial sequences with a Q <25 were removed and the retained sequences were then assigned to Operational Taxonomic Units (OTUs) based on 97% similarity with the SILVA reference database after filtering chimeras using VSEARCH [39] with the ribosomal database project (RDP database) [40]. Low confidence OTUs was removed. To correct the sampling effect, the number of sequences was established at 19.840.

Fungal raw reads were trimmed for adapters and low quality reads with the Trimmomatic v 0.38 program [41], setting the quality cutoff to 20 in 24bp sliding windows. Trimmed reads were assembled using the paired-end read merger (PEAR) program v 0.9.10 [42]. Chimeras were removed using VSEARCH from the QIIME pipeline using the UCHIME reference dataset. OTUs were assigned based on 97% similarity using the open reference OTU picking protocol implemented in the QIIME toolkit. Taxonomy was assigned using the UNITE database [43]. Low abundance OTUs (OTUs with less than 3 reads) were removed.

The sequences were uploaded to the European Nucleotide Archive (ENA) with the study accession code PRJEB38121.

2.5. Fungal pathogen detection by qPCR

Real-time PCR was performed to quantify the number of ITS copies in the soil DNA using a 7500 Fast Real-Time PCR system (Applied Biosystems). The reaction mixtures (15 µL) contained a final concentration of 1× TaqMan Universal Master Mix II, no UNG (Applied Biosystems), 0.3 µm of each primer, 0.1 µm of TaqMan probe, 0.1 mg mL⁻¹ of bovine serum albumin (BSA), 3 µL of DNA template and nuclease-free water. The PCR program consisted of an initial denaturation step at 95 ºC for 10 min, followed by 40 cycles at 95 ºC for 10 s and at 60 ºC for 40 s, and a final step at 50 ºC for 2 min. Three real-time PCRs were carried out for each DNA sample. The amplification results were analyzed with 7500 Fast Real-time PCR software v.2.0 (Applied Biosystems). Fungal path-

ogen detection and quantification was performed using the Vegalert qPCR quantitative kits for curcubits [*Alternaria spp. (ALT), Rhizoctonia solani (RSO),* and *Fusarium oxysporum (FOX)*] (Microgaia Biotech S.L, Murcia, Spain).

2.6. Statistical analysis

The normality and homogeneity of variance assumptions were evaluated using Shapiro-Wilk and Levene's tests. For mean comparison between cropping systems, one-way analysis of variance (ANOVA) was performed, followed by Tukey's honestly significant difference (HSD) post-hoc test if the effects were significant. Where conditions for homoscedasticity were not met, we used Welch's test followed by the 'pairwise.t.test' function with P adjusted by the Bonferroni-Holm method for multiple comparisons [44]. Non-parametric Kruskal-Wallis tests were used when normality assumptions were not fulfilled. When such test statistics were significant, Kruskal–Wallis multiple comparison Z-value tests were performed using the 'dunnTest' function with P adjusted by the Benjamini-Hochberg method in the FSA package [45]. Principal Component Analysis (PCA) was conducted as an unsupervised learning dimension reduction technique to visualize the cohesion and separation of the three cultivation systems. From the outcomes reported by the FactoMineR package [46], a PCA biplot was generated using the factoextra package [47] to assess the contribution of each parameter to the component loading.

For both bacterial and fungal communities, the rarefaction curves and the Chao1 and Shannon diversity indexes were calculated using the R packages iNEXT [48] and vegan [49]. The effects of the cultivation systems on such indexes were evaluated by one-way ANOVA. Significant differences were tested by Tukey's HSD test. Violin plots were generated to show the distributional shape of each index across all soil samples grouped according to the cropping system.

Similarity Percentages (SIMPER) analysis was conducted using the 'simper' function of the vegan package to identify the parameters that most contributed to pairwise differences between cropping systems at the phylum level. PERMANOVA was conducted to test the differences among the cropping systems if the homogeneity of variance assumption was met, and Analysis of Similarities (ANOSIM) was carried out if not.

A Linear Discriminant Analysis (LDA) Effect Size (LEfSe) (Galaxy community hub) under the default parameters was implemented to identify differentially abundant groups among the three cropping systems [50,51].

In order to visualize and to test whether the microbial community structures (OTUs) of the three cropping systems were distinct, Principal Coordinate Analysis (PCoA) and Permutational Multivariate Analysis of Variance (PERMANOVA) were conducted based on the Bray-Curtis distance, using the 'betadisper' and 'adonis' functions in the vegan package with 999 permutations. The soil microbial community composition was ordinated applying Non-Metric Multidimensional Scaling (NMDS) with the Bray-Curtis dissimilarity matrices using the 'metaMDS' function in the vegan package. During the NMDS analysis, the relationships between soil properties and soil microbial community were assessed by the 'envfit' function available in the vegan package.

3. Results

3.1. Effects of different cropping systems on soil physico-chemical properties, soil pathogens and crop yield

Univariate analysis showed that Org_M had a significantly higher pH (8.70) than Conv (8.39), with no significant differences with Org_C (8.47) (Table 2). The TOC, TN and NH_4^+ contents were significantly higher in Org_C than in the other two systems, while the NO_3^- content was significantly higher in Conv than in Org_C. POC showed significantly higher values in Org_M than in Org_C. Fe, Mn and B showed significantly higher values in Org_C and Conv than in Org_M. Mg was significantly higher in Org_C than in Org_M, and K was significantly higher in Org_C than in Conv (Table 2). The Conv system showed the significantly highest amount of total pesticides (TP).

Table 2. Effects of the three cropping systems on soil properties.

Soil properties	Cropping system			Anova	Kruskal-Wallis
	Conv	Org_C	Org_M		
pH	8.39±0.17 b	8.47±0.14 ab	8.70±0.10 a	*	-
EC (dS m^{-1})	0.54±0.15	0.52±0.13	0.38±0.04	-	ns
TOC (g kg^{-1})	11.49±0.28 ab	15.64±3.37 a	9.01±3.49 b	**	-
TN (g kg^{-1})	1.13±0.19 b	1.59±0.34 a	0.93±0.24 b	**	-
POC (g kg^{-1})	2.67±0.72 ab	2.20±0.55 b	4.03±1.40 a	*	-
NH$_4^+$ (mg kg^{-1})	0.10±0.23 b	1.33±0.15 b	0.00±0.00 a	-	**
NO$_3^-$ (mg kg^{-1})	53.04±28.27 a	11.86±7.10 ab	27.00±13.57 b	-	*
Bulk density (kg dm^{-3})	1.24±0.06	1.27±0.07	1.34±0.09	ns	-
SWW (cm^3 cm^{-3})	0.12±0.01	0.12±0.02	0.12±0.03	ns	-
SWFC (cm^3 cm^{-3})	0.22±0.01	0.25±0.03	0.22±0.02	ns	-
CEC (cmol kg^{-1})	14.82±0.86	17.47±4.13	12.76±2.20	-	ns
CaCO$_3$ (%)	44.65±2.71	45.54±7.57	47.03±1.92	-	ns
FMA (cm^3 cm$^{3-1}$)	0.17±0.03	0.20±0.06	0.19±0.03	-	ns
Ca (cmol kg^{-1})	8.44±0.83	10.03±2.40	7.19±1.49	-	ns
Mg (cmol kg^{-1})	3.54±0.11 ab	4.39±1.09 a	3.13±0.54 b	*	-
K (cmol kg^{-1})	0.62±0.15 b	0.85±0.17 a	0.78±0.06 ab	*	-
Na (cmol kg^{-1})	2.12±0.32	2.19±0.86	1.64±0.23	ns	-
P (mg kg^{-1})	20.15±5.24	14.65±7.71	14.33±7.48	ns	-
Cu (mg kg^{-1})	2.17±0.74	3.17±0.81	2.19±0.75	ns	-
Zn (mg kg^{-1})	4.75±2.99	5.46±1.65	4.48±0.91	ns	-
Fe (mg kg^{-1})	6.19±2.91 a	6.97±2.69 b	2.99±1.24 a	-	*
Mn (mg kg^{-1})	9.47±1.03 a	7.91±2.37 a	4.66±0.36 b	***	-
B (mg kg^{-1})	1.68±0.11 a	1.94±0.28 a	1.31±0.17 b	**	-
TP (ng g^{-1})	232.00±146.54 a	13.36±9.11 b	6.08±5.21 b	-	**
ALT (log copy ITS g^{-1} soil)	4.24±0.32 a	4.33±0.49 a	2.05±1.88 b	-	**
RSO (log copy ITS g^{-1} soil)	0.67±1.51	2.72±1.61	2.02±1.86	-	ns
FOX (log copy ITS g^{-1} soil)	3.08±1.73 a	3.30±0.44 a	0.63±1.41 ab	-	*

EC, Electrical Conductivity; TOC, Total Organic Carbon; TN, Total Nitrogen; POC, Particulate Organic Carbon; SWW, Soil Wilting Point; SWFC, Field capacity; CEC, Cation Exchange Capacity; FMA, Actual field soil moisture; Available (Ca, Mg, K, Na, P, Cu, Zn, Fe, Mn, B); TP, Total Pesticides; ALT, Alternaria spp.; RSO, Rhizoctonia solani; FOX, Fusarium oxysporum. Conv, Conventional system; Org_C, Organic cultivation with sheep manure compost and compost tea; Org_M, Organic cultivation with sheep manure. Values (mean±standard deviation n=5) followed by different lower letters correspond to significant differences between cultivation systems (Tukey's test or pairwise t-test by groups); (ns) non-significant differences between cultivation systems. (-) the test does not proceed; significant levels: *** $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

The abundance of *Alternaria* spp. (ALT) was significantly lowest in Org_M, while *Fusarium oxysporum* (FOX) was significantly lower in Org_M than in Conv and Org_C; there were no significant differences in *Rhizoctonia Solani* (RSO) among cropping systems (Table 2).

The biplot obtained reflected differences among the three cropping systems (Figure 1). Org_C was associated with TOC, TN, B, Mg and NH4+, contrary to Org_M negatively correlated with pH (Figure 1). The Conv system was positively correlated with total pesticide content.
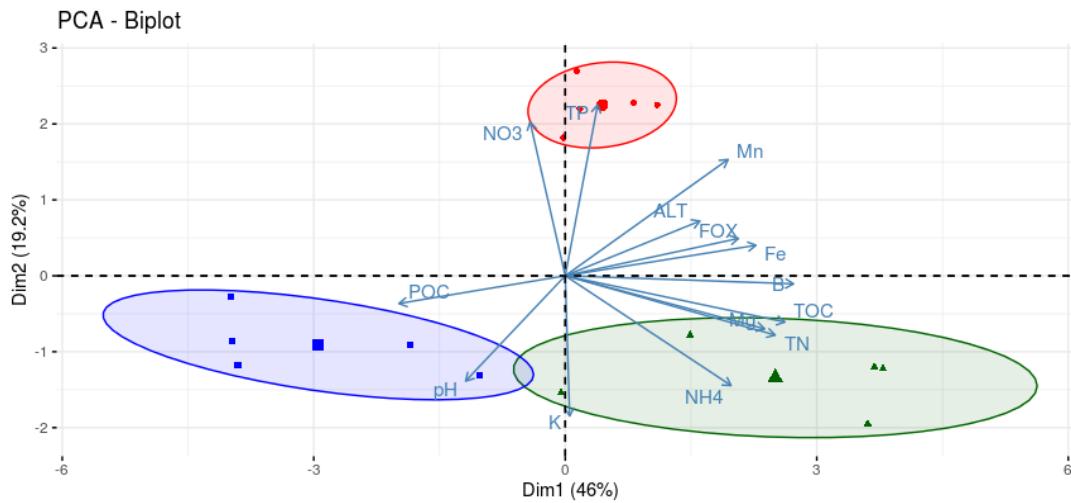
Figure 1. Principal Component Analysis (PCA) of the soil properties. The two first principal components are shown on the x- and y- axes, respectively. PCA scores represent soil samples coloured indicating the corresponding cultivation system. Ellipses plotted represent 70% confidence intervals around the bary centres for samples classified by each cultivation system. TOC, Total Organic Carbon; TN, Total Nitrogen; POC, Particulate Organic Carbon; Mg, Exchangeable Mg; Fe, Bioavailable Fe; Mn, Bioavailable Mn; B, Bioavailable B; TP, Total Pesticides; ALT, *Alternaria spp.*; FOX, *Fusarium oxisporum*; Conv, Conventional system; Org_M, Organic cultivation system with sheep manure; Org_C, Organic cultivation system with sheep manure compost and compost tea. (n=5, per cropping system).

No significant differences were found for cabbage yield among the three cropping systems (Figure 2).
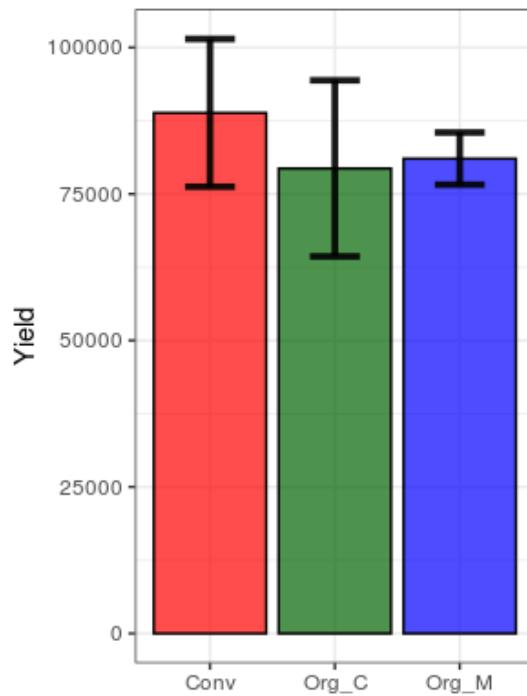
Figure 2. Crop yield (kg ha^{-1}) in the three cropping systems. Error bars represent mean±SD (n=5). No significant differences were found among the three cultivation systems (Kruskal-Wallis test, P > 0.05). Conv, Conventional system; Org_M, Organic cultivation system with sheep manure; Org_C, Organic cultivation system with sheep manure compost and compost tea.

3.2. Effects of different cropping systems on soil microbial diversity

A total of 592,250 16S sequences (clustered into 18,533 OTUs) for bacteria and 1,186,964 ITS sequences (clustered into 611 OTUs) for fungi were obtained from all soil samples. Rarefaction curves showed (a coverage value of 0.84-0.88 for bacteria and 0.99 for fungi Figure S1. Different microbial indices were calculated for microbial community (Figure 3; Table S2). The Chao1 index showed no significant differences in the bacterial and fungal communities among the three cropping systems (Figure 3; Table S2). The Shannon index showed no differences for the bacterial community, but did show significant differences for fungi, which had significantly higher values in Conv and Org_M than in Org_C (Figure 3; Table S2).

(A)   *(B)*

(C)   *(D)*
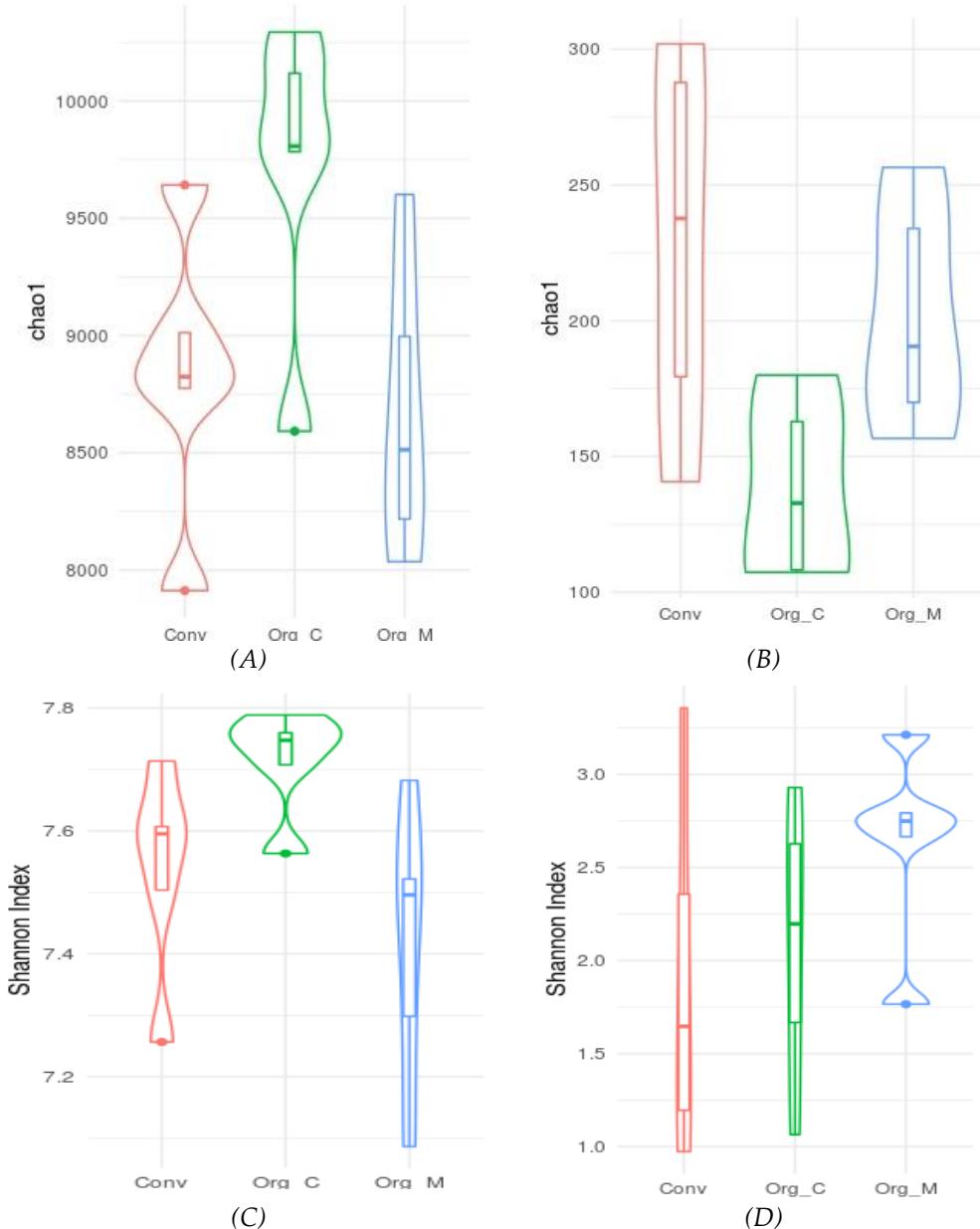
Figure 3. Violin plots displaying of diversity indexes in the three cultivation systems. The distributional features of the data are depicted by the kernel density trace overlaid the descriptive statistics (median and whisker range from 25% to 75%) represented by a boxplot. Chao1 for bacterial (A) and fungal community (B), and Shannon index for bacterial (C) and fungal community (D). Conv, Conventional system; Org_M, Organic cultivation system with sheep manure; Org_C, Organic cultivation system with sheep manure compost and compost tea. (n=5, per cropping system).

PCoAs of the bacterial (Figure 4A) and fungal (Figure 4B) microbial communities showed significant differences among the different cropping systems, that differences

were confirmed by PERMANOVA (PERMANOVA: F = 1.792, P= 0.006; F = 7.649, P = 0.001 respectively).


(A)

(B)
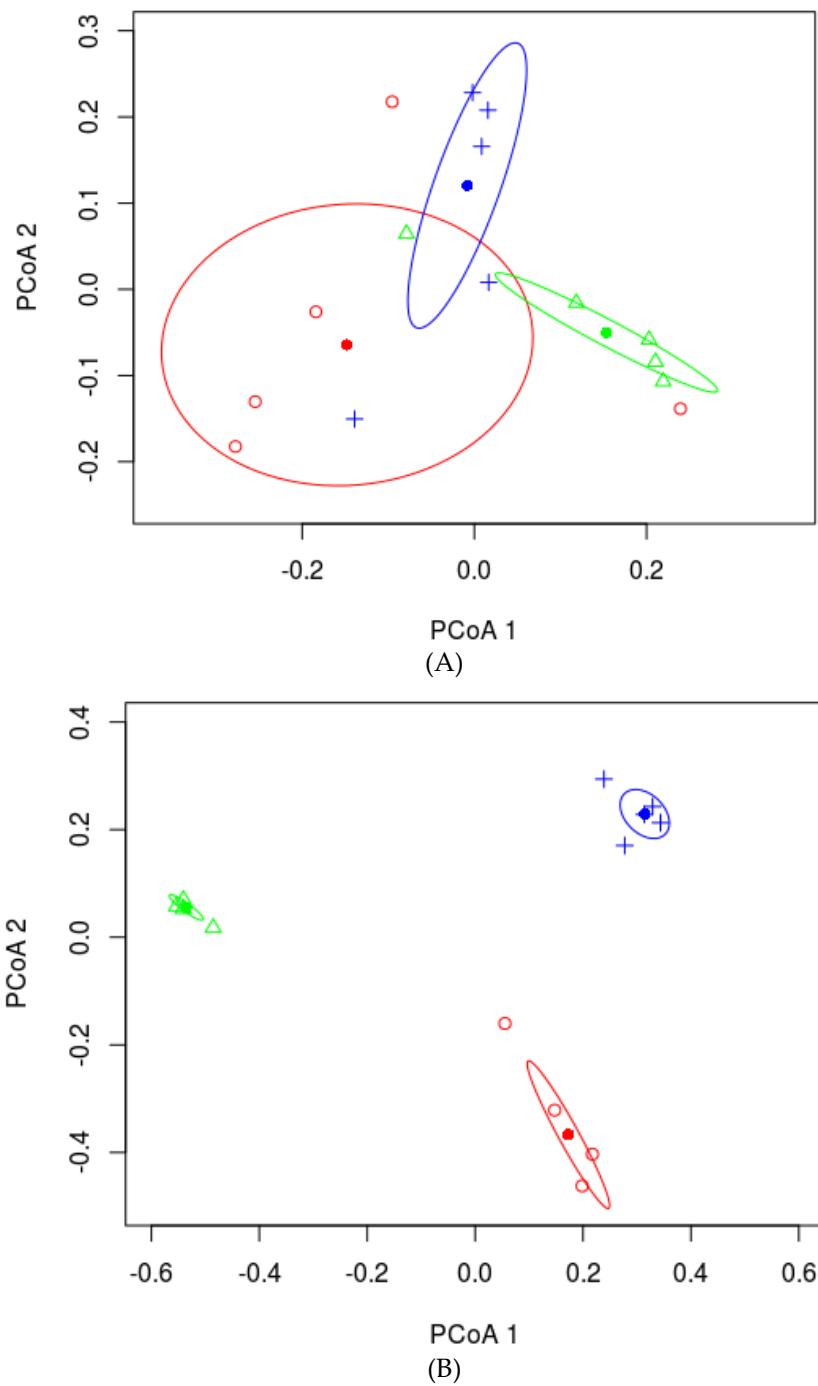
Figure 4. Principal Coordinate Analysis (PCoA) of the (A) bacterial (B) fungal community structure between the three cropping systems. Different colours and shapes represent the different points of the group, Conv in red colour and circle shape, Org_C in green colour and triangle shape and Org_M in blue colour and cross shape. PCoA display group centroids and dispersions. Conv, Conventional system; Org_C, Organic cultivation with manure compost and tea compost; Org_M, Organic cultivation with manure. (n=5, per cropping system).

3.3 Effects of different cropping systems on bacterial and fungal community composition

The main bacterial and fungal taxa found in the different cropping systems are shown in Figure 5, 6 and 7. The most dominant phyla under the different cropping systems were Proteobacteria (42% in average), Actinobacteria (16%), Bacteriodetes (12%) and Acidobacteria (7%) (Figure 5A; Table S3). No significant differences were found among the three cropping systems using Bray-Curtis dissimilarity at phylum level (PERMANOVA: F = 0.821; P = 0.562). SIMPER pairwise comparisons showed that the Proteobacteria, Gemmatimonadetes, Bacteriodetes and Actinobacteria accounted for 75% of the overall dissimilarities between Conv and Org_M or Org_C.
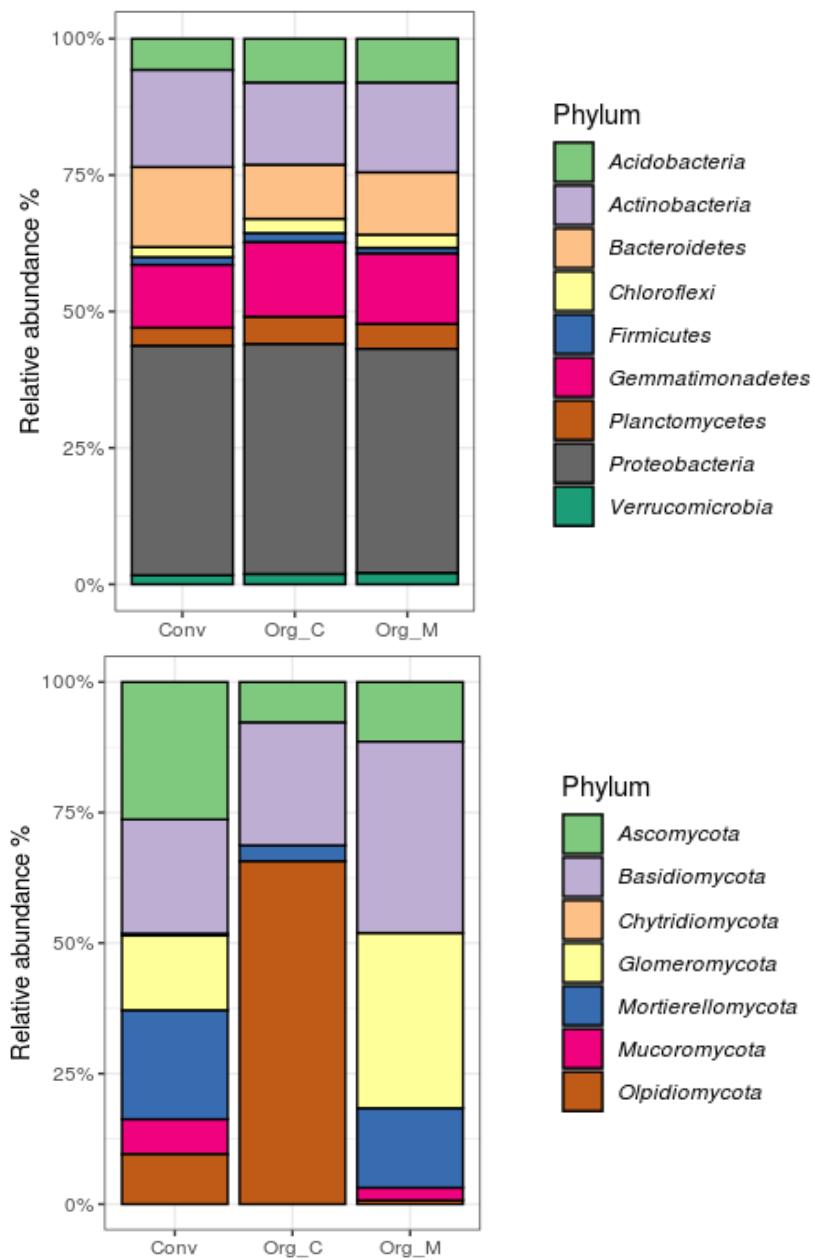
Figure 5. Relative abundance (>1%) of (A) bacterial (B) fungal phylum of the three cropping systems. Bar values are mean n=5. Conv, Conventional system; Org_C, Organic cultivation with manure compost and tea compost; Org_M, Organic cultivation with manure.

The most abundant fungal phylum was Basidiomycota (27% on average), followed by Olpidiomycota (25%), Ascomycota (15%), Glomeromycota (15%) and Mortierellomycota (13%) (Figure 5B; Table S4). Significant differences were found between the three cropping systems (PERMANOVA: F = 4.83, P = 0.003). The highest relative abundance of

Basidomycota and Glomeromycota was observed in Org_M, while Olpidiomycota showed the highest abundance in Org_C. The highest abundance for Ascomycota and Mortierellomycota was found for the Conv system, followed by Org_M and Org_C (Table S4). SIMPER showed that Glomeromycota, Basidiomycota and Ascomycota accounted for 73% of the dissimilarities between Conv and Org_M, while Olpidiomycota, Basidiomycota and Ascomycota accounted for 74% of dissimilarities between Conv and Org_C.

LEfSe was conducted to identify the taxa that display significant differences among the three cropping systems. For bacteria, the Conv system had fifteen differential taxa while Org_C had seven and Org_M had eleven (Figure 6A and 7A). At the genus level, *Nesterenkonia* (Actinobacteria), *Galbibacter* and *Gramella* (Bacteroidetes), *Limnobacter*, *Pseudoalteromonas* and *Pantoe* (Proteobacteria) were the most differential taxa in the Conv system. The genomic features in the organic systems identified the genera *Aciditerrimonas* and *Isoptericola* (Actinobacteria), *Nibribacter* (Bacteroidete), *Haliangium* (Proteobacteria) and *Terrimicrobium* (Verrucomicrobia) as important taxonomic contributors for Org_M, and the genera *Dinghuibacter* and *Turibacter* (Bacteroidete), *Piscibacillus* and *Planifilium* (Firmicutes) and *Roseibacillus* (Verrucomicrobia) as important taxonomic contributors for Org_C (Figure 6A and 7A). For fungi, the Conv system showed two differential taxa; among the organic systems, Org_C showed two differential taxa and Org_M seventeen (Figure 6B and 7B). The genus *Sporobolomyces* (Basidiomycota) was the only genomic feature in the Conv system. For the organic systems, we found the genera *Wallemiales* (Basidiomycota), *Funneliformis* (Glomerales), *Melanoleuca* (Basidiomycota), *Alternaria* and *Paraphaeosphaera* (Ascomycota) in Org_M, and the genus *Candida* (Ascomycota) in Org_C.


(A)

(B)
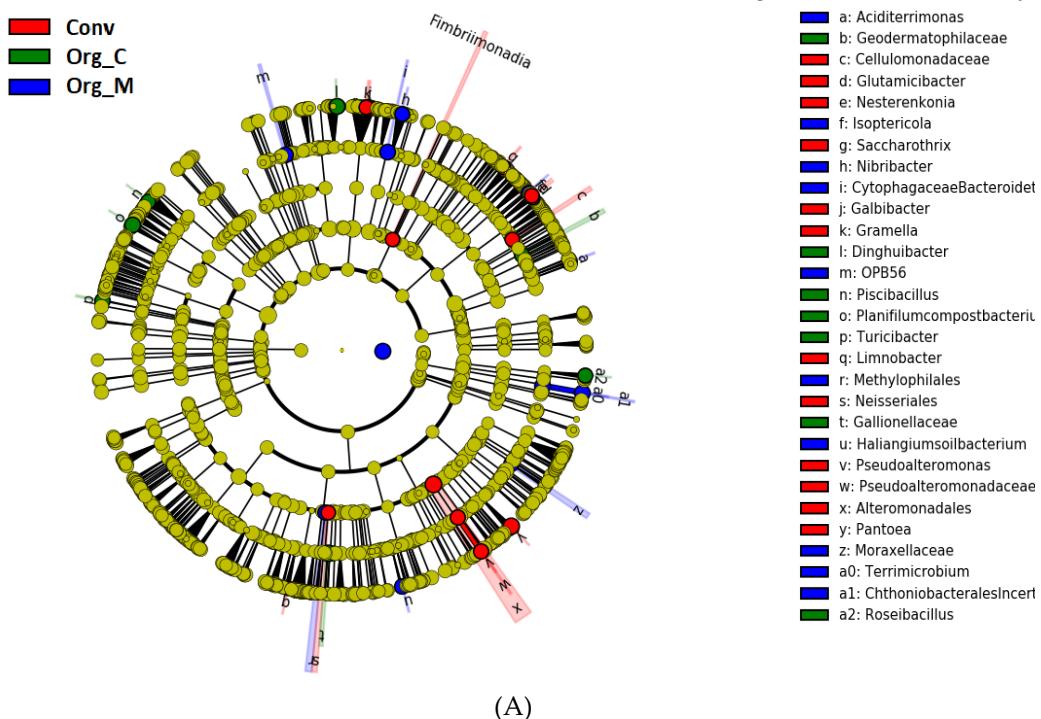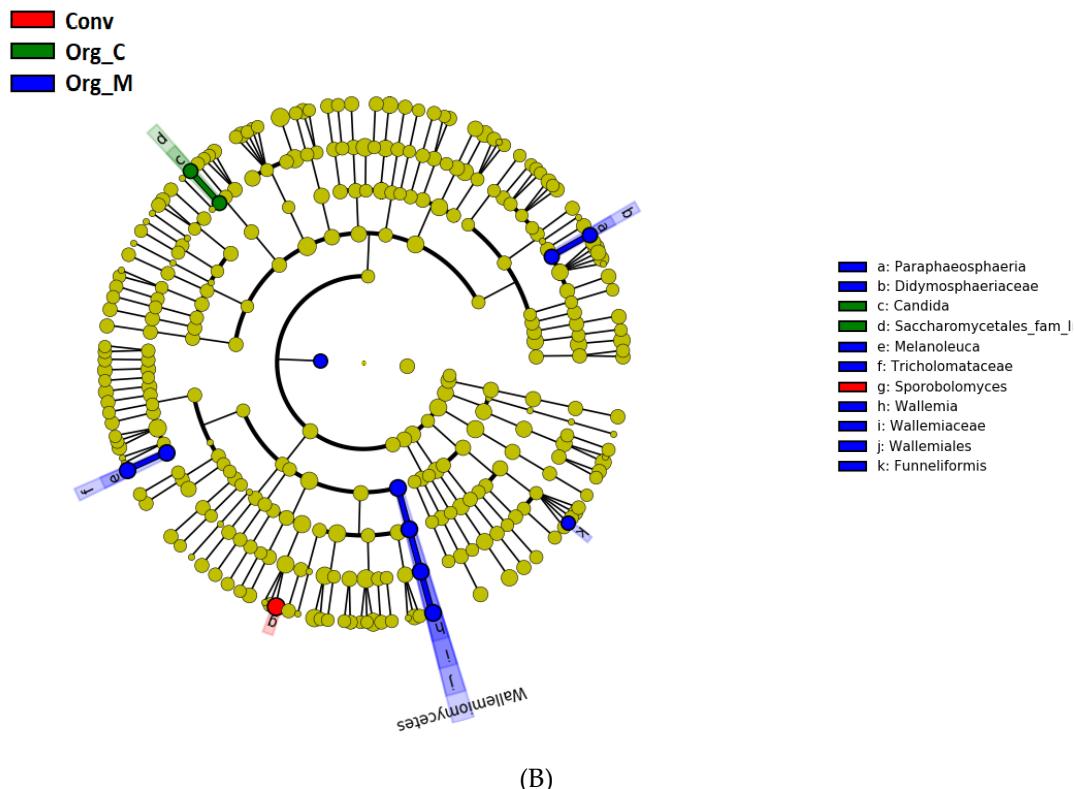
Figure 6. Taxonomic cladogram obtained from the LEfSe of (A) 16S rDNA and (B) ITS. Taxa of microorganisms are highlighted by colored circles and shaded areas (Conv, Org_C and Org_M are shown in red, green and blue respectively). Each circle represents a taxa and each circle′s diameter reflects the abundance of that taxa in the community. Conv, Conventional system; Org_C, Organic cultivation with manure compost and tea compost; Org_M, Organic cultivation with manure. (n=5, per cropping system)
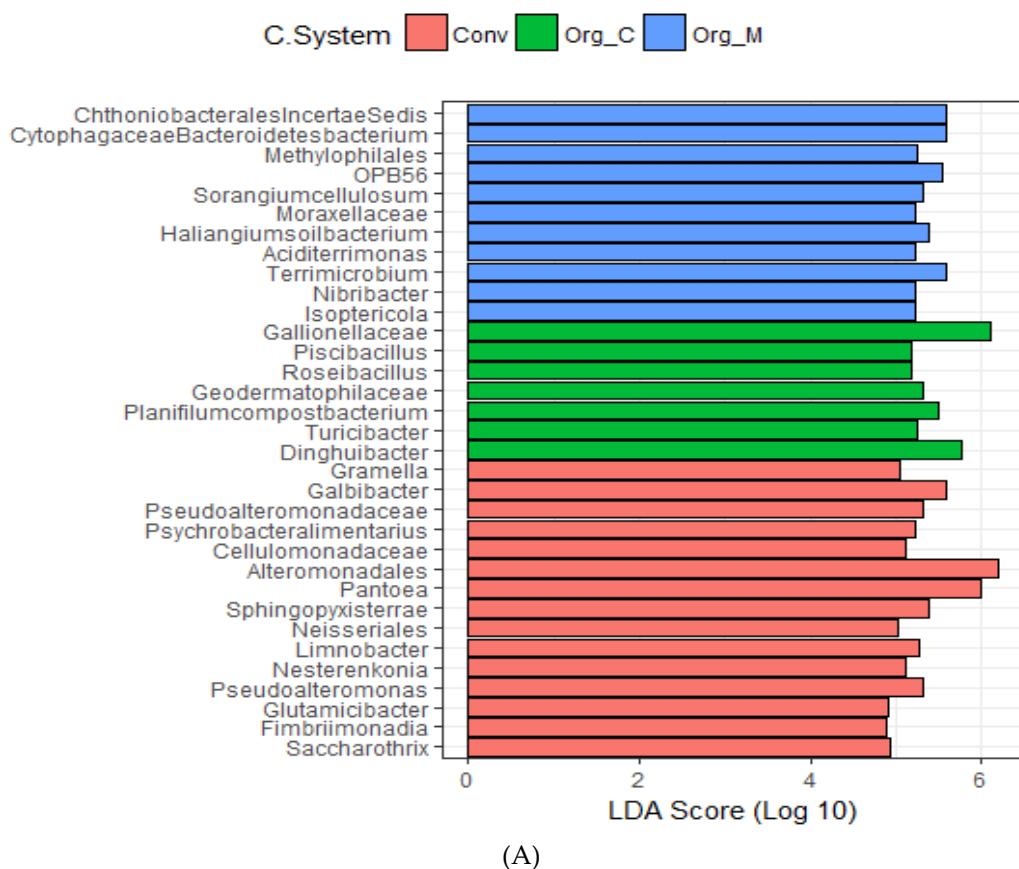
(A)
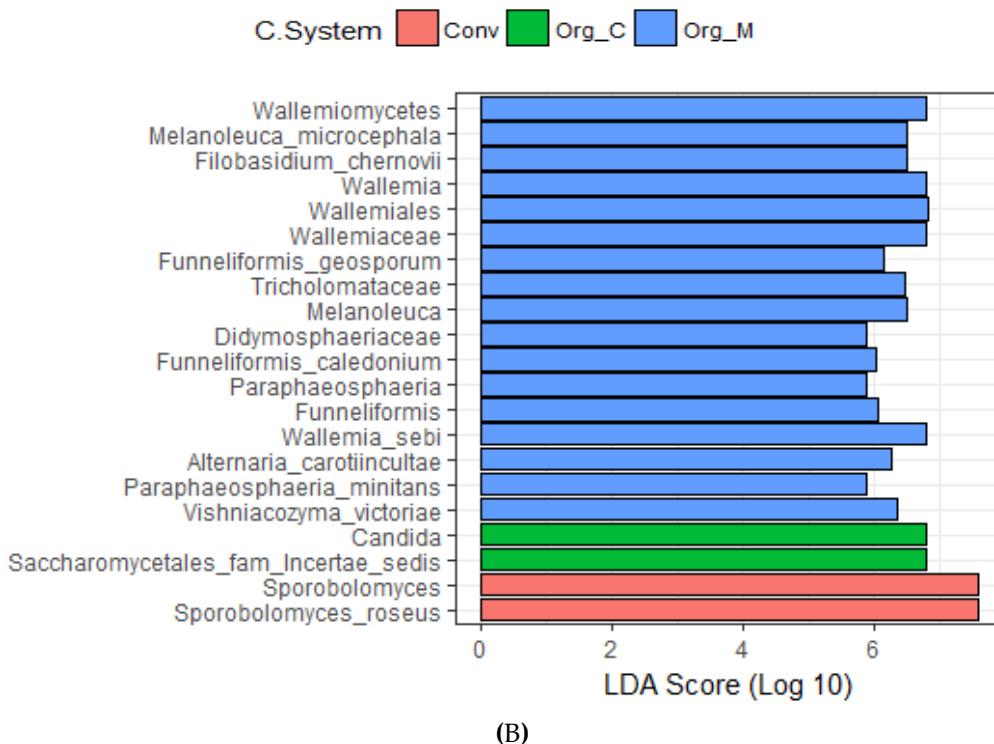
(B)

Figure 7. Linear Discriminant Analysis Effect Size (LEfSe) analysis showing (A) bacterial (B) fungal microbiota changes between the three cropping systems.. Conv, Conventional system; Org_M, Organic cultivation with manure; Org_C: Organic cultivation with manure compost and tea compost. (n=5, per cropping system).

3.4. The relationship between the microbial community and soil properties

A nonmetric multidimensional scaling (NMDS) was assayed for establishing the relationship of bacterial and fungal communities with the significant soil parameters. For bacteria, TOC, TN, $NH4^+$, Mg and, B were the soil properties that showed a significant effect on bacterial community composition (Table S5). For fungi, however, no significant correlation was found (Table S6).

A Spearman correlation analysis between soil properties and bacterial and fungal genera showed a significant correlation between *Nesterenkonia* and total pesticide (0.68**); *Gramella* and total pesticide (0.52*); *Pseudoalteromonas* and *Pantoe* with total pesticide (0.56*); *Sporobolomyces* and total pesticide (0.80); *Planifilium*, *Dinghuibacter*, *Turicibacter*, *Piscibacillus, Melanoleuca, Filobasidium* and *Candida* showed a high correlation with NH4+ (0.60*, 0.66**, 0.59*, 0.74**, -0.77**, -0.76** and 0.70** respectively); *Aciditerrimonas, Roseibacillus* and *Nibribacter* showed high correlation with TOC (0.82***, 0.74**, -0.65** respectively) and *Aciditerrimonas* with total nitrogen (0.64**); *Terrimicrobium* with NH4* (-0.58*); *Wallemia* with both pathogens *Alternaria spp* (-0.77**) and *Fusarium oxysporum* (-0.56**) and with NH4* (-0.66*).

4. Discussion

Organic and conventional farming are nowadays defined based on differences regarding fertilization, plant cultivation and soil management. Our three cropping systems have the same soil type and climate, are located in close proximity and have undergone the same crop rotation. Therefore, it is likely that the differences observed in soil properties and microbial community are probably due to crop systems. Results highlight that once organic systems are stabilized over time, production can be as high as that of conventional systems if the soil and crops are effectively managed and nutrient availability is ensured [52,53].

Differences among the cropping systems related to soil properties indicated that Conv system showed higher NO_3^- levels, probably due to intensive chemical fertilizer and pesticide use, and the addition of nitric acid in fertigation [54,55]. To the contrary, Org_C was characterized by higher soil nutrient availability and organic carbon due to the long-term incorporation of compost, a stable organic matter with a low decomposition rate compared to manure. Compost treatments increase the long-term stabilization of organic matter in the soil and hinder nutrient leaching by rapid mineralization, making nutrients more available for plant uptake [56]. The highest pH showed by Org_M can be explained by the buffering capacity from bicarbonates and organic acids in the manure, with no addition of nitric acid as used in Conv to decrease pH [57]. The higher organic carbon content in Org_C with higher stabilization may have contributed to the slightly lower pH owing to the greater presence of organic acids [58,59]. The addition of compost instead of manure may therefore provide positive effects in basic soils, contributing to a decrease in pH and making nutrients more available.

It is interesting to highlight that soil parameters such as TOC and some nutrients like TN, Mg and B were associated with changes in soil bacterial community, although this association was not observed in the fungal community. Similar results were observed by Yang et al. [60] and Zhang et al. [61] who found a link between the bacterial community and soil chemical properties. Organic carbon is considered necessary to alter microbial community regardless other factors [62] and is well known that compost enhance carbon and nitrogen in soils for microbial communities [63]. Also, Vera et al. [64] observed that the B content in the soil was a determinant property explaining changes in the bacterial community in an agricultural soil. According to Vera et al [65] organic matter had the key influence on potential microbial action with high B doses and our findings could support that influence, since some key microorganisms from soil organic cropping system had a high correlation with boron. Although the bacterial community structure changed in response to the different cropping systems, this was not associated with significant variations in alpha diversity [66]. It was contrary to lower values expected in conventional systems due to the adverse effects of agrochemicals [67–69]. Moreover, a significant higher fungal diversity was observed in Conv system probably due to the increase in nutrient availability observed by Geisseler and Scow [70] and Leff et al. [71], and the breakage of fungal hyphae by tillage [72].

Legacy effects of cropping systems occur in specific microbial groups and cannot be resolved by determining the diversity of the entire microbial community, as shifts in some groups might be compensated for shifts in others [73]. Most importantly, our results showed that there are effects of different cropping systems on the soil microbial community structure. Indeed, the impact of the cropping system is considered to be stronger than any potential spatiotemporal variations [74]. The effect of the different cropping systems was not reflected in the dominant bacterial phyla, contrary to that observed by Lupatini, et al. [73] or Moreno-Espíndola et al [75]. Hartman et al. [74] observed that differences in the bacterial community between organically farmed and conventionally managed soils under integrated fertilization were smaller. Contrary to the bacterial community, the fungal microbial composition did change significantly among the three cropping systems. The conventional system showed a greater abundance of Ascomycota considered to be a key player in the decomposition of soil organic matter and dominates the active fungal community through its involvement in root exudation, assimilation and soil organic matter degradation [76,77] and, Mortierellomycota that can be saprophytic on the residual limbs of plants and animals and decompose their remains [78].

Basidiomycota and Glomeromycota accounting the greatest dissimilarity in Org_M compared with conventional cropping system and previous studies showed Basidiomycota, which are the main decomposer of plant litter [79] was increase in soils with manure since it provide an appropriate environment for Basidiomycota [80] However, chemical fertilizer can cause the loss of that environment and hinder the development of this phyla [80]. High abundance of Glomeromycota in Org_M can form arbuscular my-

corrhiza with plants and absorb nutrients directly, particularly P uptake of plant roots [81], promoting plant growth, and enhances plant resistance to various pathogens [82], so it could be possible that its high abundance is related to the lower abundance of both pathogens *Fusarium oxysporum* and *Alternaria spp*. and the nearly non-existant abundance of Olpidyomicota (*Olpidium*). Contrarily, Olpidyomicota was more abundant in Org_C, in which no Glomeromycota was found. However, the possible infection of plants by these pathogens in subsequent crops could depend on the amount of pathogens necessary to cause disease and the environmental factors [83]. Carini et al. [84] discovered that up to 50% of the microbial nucleic acid sequences in environmental samples could correspond to dead and inactive biomass. So, quantification by RNA would therefore provide a more meaningful assessment of cellular viability and plant infection [83].

The presence of several differentially abundant taxa among cropping systems provides information on soil microbiota responses to different agricultural management practices [85]. According to LEfSe analysis, the long-term application of pesticides in Conv system favored the greater existence of microorganisms associated with the pesticides, such as *Nesterenkonia, Galbibacter, Gramella, Limnobacter, Pseudoalteromonas, Pantoe* and *Sporobolomyces.* Agrochemicals have the potential to inhibit or eliminate certain groups of microbes and select members adapted to or able to grow under conventional farming practices [86]. *Pantoea* comprises many versatile species with different functions like the degradation of herbicides and other toxic compounds, and some isolates are antibiotic producers [87]. Some *Pseudoalteromonas* strains can produce bioactive compounds [88], and Sporobolomyces is a yeast capable of pesticide degradation [61]. Under Org_C, *Turicibacter, Dinghiubacter, Planifilium, Roseibacillus, Piscibacillus* and *Candida* were more abundant. High levels of ammonium in this organic framing treatments could favor the presence of these genera. Org_M-related genera were *Aciditerrimonas, Isoptericola, Nibribacter, Haliangium, Terrimicrobium, Funneliformis, Wallemia, Melanoleuca* and *Filobasidium. Aciditerrimonas* is related to ferrous-ferric redox [89] and showed a correlation with TOC and NH_4^+. *Haliangium* is a genus in which some species are producers of haliangicins, known antifungal compounds [90], or *Wallemiales,* which have been observed in organic cultivations in other studies [91] and have shown a strong negative correlation with *Alternaria spp* and *Fusarium Oxysporu*m. This suggests that an increase in this genus could favor a decrease in some phytopathogens [92], as we observed in this cropping system.

5. Conclusions

This work showed that properly long-term organic systems can contribute to keep high and stable crops. Where, bacterial and fungal communities changed under those organic cropping systems compared to conventional system. Analyses showed that fungal communities were more sensitive to cropping systems than bacteria due to changes from phylum level, while, bacteria changed in other more specific taxonomy level. Also, LEfSe analysis revealed some key microorganisms associated to different cropping which could had a special function in soils since in Conv system the key microorganisms were principally associated with pesticides and in organic systems microorganisms have a direct beneficial effect on plant.

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