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TESIS DOCTORAL

ESTUDIO BIOINFORMÁTICO DEL MICROBIOMA DEL SUELO BAJO DIFERENTES SISTEMAS DE CULTIVO

Presentada por Jessica Cuartero Moñino para optar al grado de Doctora
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ESCUELA INTERNACIONAL DE DOCTORADO

DOCTORAL PROGRAMME IN ADVANCED TECHNIQUES FOR RESEARCH AND DEVELOPMENT IN FOOD AND AGRICULTURE

PhD THESIS

BIOINFORMATIC STUDY OF THE SOIL MICROBIOME UNDER DIFFERENT CROPPING SYSTEMS

Presented by JESSICA CUARTERO MOÑINO

to the Technical University of Cartagena in fulfilment of the thesis requirement for the award of PhD

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To my family

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- **Paper 1.-** Cuartero, J., Özbolat, O., Sánchez-Navarro, V., Egea-Cortines, M., Zornoza, R., Canfora, L., Orrù, L., Pascual, J.A., Vivo, J.M. and Ros, M., 2021. Changes in Bacterial and Fungal Soil Communities in Long-Term Organic Cropping Systems. *Agriculture*, 11(5), p.445.

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  Cuartil: 1-Agriculture, Multidisciplinary

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Resumen

Mantener la producción o, incluso aumentarla, es uno de los retos de esta época, ya que la población aumenta cada día. Sin embargo, la producción está disminuyendo por muchas razones, siendo una de ellas la degradación del suelo. Hay muchos factores que provocan esta degradación de los suelos como: la erosión, la pérdida de materia orgánica, el viento, el agua y, muy especialmente, las prácticas agrarias intensivas, que incluyen el uso de: pesticidas, herbicidas y fertilizantes químicos. Para evitar este daño al suelo, están surgiendo propuestas más sostenibles.

Para estudiar la calidad del suelo generalmente se determinan algunos parámetros fisicoquímicos, químicos y biológicos, aunque las nuevas técnicas moleculares (como la secuenciación de última generación), están ganando fuerza para evaluar la calidad del suelo, debido al papel crucial que juegan los microorganismos en mantener la salud del suelo, participando en los ciclos bioquímicos (como el ciclo del nitrógeno) y, en consecuencia, en la producción agraria.

Actualmente están surgiendo herramientas computacionales para analizar esos datos y sus relaciones, a través de técnicas estadísticas multivariantes conducidas dentro del campo de la bioinformática. De hecho, existen diversos recursos bioinformáticos para profundizar en el estudio de la estructura microbiana, su funcionalidad y las interconexiones entre la comunidad microbiana así como las propiedades del suelo.

Por lo tanto, el **objetivo de esta tesis** es evaluar la respuesta de los microorganismos del suelo a través del uso de herramientas bioinformáticas, su análisis estadístico y las relaciones de estos con los parámetros fisicoquímicos, físicos, químicos, biológicos y la producción de los cultivos bajo practicas **de cultivo sostenible**.

Los tres trabajos que han sido publicados son:

a) Cambios en las comunidades bacterianas y fúngicas en sistemas de cultivo orgánico de largo plazo. *Agriculture* (https://doi.org/10.3390/agriculture11050445).

b) Cambio en las interacciones y especializaciones en las comunidades bacterianas del suelo tras una aplicación a largo plazo de compost, que incrementó la presencia de genes del ciclo del nitrógeno en el suelo. *Agronomy* (https://doi.org/10.3390/agronomy12020316).

c) Un año de cultivos asociados de melón:caupí mejora los nutrientes y cambia las comunidades microbianas del suelo. *Agriculture, Ecosystems & Environment* (https://doi.org/10.1016/j.agee.2022.107856)

Las primeras dos publicaciones estudian el efecto de un experimento a largo plazo (10 años) donde dos cultivos orgánicos, uno con la adición de compost y té de compost y el otro con la adición de estiércol fresco, son comparados con el cultivo convencional. Para este propósito, las propiedades del suelo y las comunidades microbianas, determinadas a través de la secuenciación de los genes 16S e ITS, son estudiadas mediante el uso de técnicas estadísticas, tanto *univariantes* como *multivariantes*, y mediante el *Análisis Discriminante Lineal por Efecto del Tamaño* (LEfSe). Para profundizar en la funcionalidad, estructura y conexiones microbianas se aplican avanzados algoritmos bioinformáticos como la *Investigación Filogenética de Comunidades por la Reconstrucción de Estados No observados* (PICRUSt) y el *Análisis de Redes Moleculares* (MENA).

Los resultados muestran como los dos sistemas sostenibles, en particular en el que se aplica el compost, produce un aumento del carbono orgánico total, nitrógeno total y algunos micronutrientes como boro o magnesio, comparado con el convencional. Con respecto a la comunidad microbiana, aunque la α-diversidad no detectó diferencias entre los sistemas de cultivo, la β-diversidad y el algoritmo *LEfSe* son capaces de detectar cambios en las comunidades bacterianas y fúngicas, donde diferentes microorganismos se muestran asocidados a los diferentes cultivos realizados ejem. *Haliangium*, Wallemiales, *Turicibacter*, *Pantoea* o *Pseudoalteromonas*.

La construcción de redes de coocurrencia y su posterior análisis, así como la predicción de una funcionalidad potencial, muestra como la incorporación de compost aumentó la modularidad, permitiendo que la comunidad hospedara un mayor número de nichos, lo que facilitaría la respuesta de la comunidad microbiana a cambios en factores externos. Además, la funcionalidad potencial revela como la aplicación de compost aumenta la fijación potencial de nitrógeno a través de los microorganismos, disminuyendo las emisiones de N_2O y aumentando el secuestro potencial de carbono por parte de microorganismos autótrofos.

La tercera publicación incluye el efecto, a corto plazo, de distintas distribuciones de cultivos asociados como melón (*Cucumis melo*) y caupí (*Vigna unguiculata*), en comparación con el monocultivo de melón y caupí, sobre las propiedades del suelo y la comunidad bacteriana obtenida a través de la amplificación del gen 16S rRNA.

Los resultados obtenidos a través de los apropiados procedimientos estadísticos *univariantes*, *multivariantes* y el algoritmo *LEfSe* usando recursos computacionales, revelan como el sistema de cultivos asociados incrementa el nitrógeno total, el carbono orgánico total, el fósforo y la producción de melón en comparación con un sistema de monocultivo, pero también revela como el cultivo asociado cambia la población bacteriana y promueve el desarrollo de algunos microorganismos beneficiosos, como *Pseudomonas*, *Bacillus*, *Sphingomonas* y *Strepmyces*.

Palabras clave

Bioinformática, Análisis estadístico, Agricultura sostenible, Cultivos asociados, Melón, Caupí, Compost, PICRUSt, Análisis de redes, LEfSe, Amplicón, Secuenciación de última generación, 16S, ITS.

Abstract

Maintaining crop yield, or even increasing, is one of the challenges of this time since every day the population increases. However, agricultural yield is declining for many reasons, being one of them, the soil degradation. There are many factors involve in this soil degradation such as erosion, loss of organic matter, the wind, the water, and most especially intensive farming practices which includes: pesticides, herbicides, and chemical fertilizers. To avoid this soil damage, many sustainable proposals have emerged.

To study soil quality, generally the measurement of some physicochemical, chemical and biological parameters are used, although new molecular techniques (such a Next Generation Sequencing), are also gaining strength to asse soil quality due to the crucial role that microorganisms play in maintaining soil health participating in biochemical cycles (such N-cycle) and, in consequence, for crop production.

Currently, there are a burgeoning development of computational tools to analyse such data, multidimensional by nature, and their interrelationships mainly through multivariate techniques conducted under the field of Bioinformatics. Indeed, specific bioinformatics resources are available to deep on the study of the microbial community structure, functionality and the interconnection between microbial community and soil properties.

Therefore, the **objective of this thesis** is to evaluate the response of soil microorganisms, through the use of bioinformatic tools and its statistical analysis, and their relationship with soil physicochemical, physical, chemical, biological parameters and crop yield when **sustainable crop systems are assayed**.

Three papers have been published:

a) Changes in Bacterial and Fungal Soil Communities in Long-Term Organic Cropping Systems. *Agriculture* (https://doi.org/10.3390/agriculture11050445).

b) Long-Term Compost Amendment Changes Interactions and Specialization in the Soil Bacterial Community, Increasing the Presence of Beneficial N-Cycling Genes in the Soil. *Agronomy* (https://doi.org/10.3390/agronomy12020316).

c) A first-year melon/cowpea intercropping system improves soil nutrients and changes the soil microbial community. *Agriculture, Ecosystems & Environment* (https://doi.org/10.1016/j.agee.2022.107856)

The first two publications study the effect of a long-term experiment (10 years) where two sustainable cropping systems with the application of compost and compost tea in one hand; and fresh manure in the other are compared with a conventional cropping system. For this purpose, soil properties and microbial communities which are determined by sequencing of 16S rRNA and ITS genes, are studied by using *univariate* and *multivariate statistical methodologies*, and *Linear discriminant analysis Effect Size analysis* (LEfSe). To deepen on functional microbial structure and microbial connections, advanced bioinformatics algorithms are applied through *Phylogenetic Investigation of Communities by Reconstruction of Unobserved States* (PICRUSt) and *Molecular Ecological Network Analysis* (MENA).

Results show that both sustainable cropping systems, in particular the one with compost, produces an increase on total organic carbon, total nitrogen and some micronutrients such as boron or magnesium compared to the conventional one. Regarding to microbial community, although α-diversity do not detected differences between different cropping system, β-diversity and *LEfSe* algorithm are able to detect changes in bacterial and fungal communities, where different microorganisms are associated with the different cultivation systems carried out e.g., *Haliangium, Wallemiales, Turicibacter, Pantoea or Pseudoalteromonas*. The co-occurrence network construction and the prediction of potential functionality show that the incorporation of compost increases the modularity, allowing the community to host greater number of niches that would facilitate the response of microbial community to environmental factors. In addition, potential functionality reveal how compost application increases bacterial nitrogen-fixing potential, decreasing N_2O emissions and increasing the carbon-sequestration

potential by autotrophic microorganisms.

The third publication includes the effect of different intercropping patterns (melon (*Cucumis melo*)/cowpea (*Vigna unguiculata*) a legume able to for nodules and fixing atmospheric nitrogen), in comparison to melon and cowpea monoculture on soil properties and bacterial community by sequencing of 16S rRNA gene.

Results through suitable *univariate* and *multivariate* statistical procedures and *LEfSe* analysis, reveal how intercropping system increases total nitrogen, total organic carbon, phosphorous and crop yield compared with melon monocrop systems, but it also reveals how the intercropping system changes the bacterial community and promotes the growth of some beneficial microorganisms such as *Pseudomonas*, *Bacillus*, *Sphingomonas* and *Streptomyces*.

Keywords

Bioinformatic, Statistical analysis, Sustainable agriculture, Organic farming, Intercropping, Melon, Cowpea, Compost, PICRUSt, Network Analysis, LEfSe, Amplicon, Next Generation Sequencing, 16S, ITS.

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Objectives

In conventional farming, chemical fertilizers, pesticides, and herbicides are commonly used. Their overuse has resulted in increased soil degradation, soil loss biodiversity and greenhouse emissions. Characteristics that can be aggravated in Mediterranean areas due to environmental conditions.

In the last decades, the concept of sustainable agriculture has been gaining popularity as an alternative to conventional farming, that not only satisfies human food and fibre needs, but also enhances environmental and natural resource quality, such as the soil. With regards to sustainable agriculture there are different kinds of managements such as: crop rotation, intercropping and organic farming, which have been growing interest among farmers.

Soil microorganisms are crucial to maintaining soil health and, in consequence, for crop yield in agricultural systems; they play a key role in different soil biogeochemical processes, soil structure, degrading contaminants and controlling soil-borne diseases. The challenge of this thesis is the understanding of the role of soil microorganisms and their complex interaction among them, through microbial relationships, their potential functionality, related to soil biogeochemical cycles, and their relationship to different sustainable agricultural.

For this purpose, microbial approaches such a high-throughput analysis of soil bacterial 16S rRNA gene and/or fungal ITS gene communities are studied through different

bioinformatic tools, and also their intercorrelations with physical, chemical and biological soil properties. Furthermore, the potential functionality of microbial communities based on 16S rRNA gene sequences from *Phylogenetic Investigation of Communities by Reconstruction of Unobserved States* (PICRUSt), especially those related to biogeochemical nitrogen cycle, are also studied to determine the effect of sustainable agriculture practices to maintain soil quality and crop yield.

As a model, two cropping systems with different sustainable agricultural managements in the Mediterranean area as alternative to the conventional agriculture management. The two studied cultivation systems are: a) a long-term crop rotation experiment with two organic managements, using leaf cabbage as a crop; b) a short-term experiment with three intercropping patterns of melon and cowpea, organic management a reduction of organic fertilization.

According to these assumptions, the **General Objective** of this doctoral thesis is to evaluate the response of **soil microorganisms**, through Next Generation Sequencing (NGS), and its relationship with **physical, physicochemical, chemical**, **biological** soil properties and **crop yield** of **two sustainable cultivation systems**, such as the use of organic amendments and the introducing of intercropping through **the use of bioinformatic tools and its statistical analysis**.

To reach this general objective, the following specific objectives are carried out:

Specific objectives

- To evaluate the effect of the cultivation systems on crop yield and physical, physicochemical, chemical, and biological soil properties, through univariate statistical analysis for selecting the informative features, and multivariant statistical analysis for exploring partitioning behaviours and interrelationships between soil properties.

- To evaluate the effect of the cultivation systems on bacterial and fungal microbial communities and taxonomies; determining differences and changes, exploring partitioning behaviours, through univariate statistical analysis to study the taxonomic differences and multivariant statistical analysis for exploring behaviours on microbial community.

- To extract co-occurrence patterns of the soil microorganisms under the studied cultivation systems, and to characterise and compare the resulting stochastic topologies for detecting ecosystem transitions and the relationships between microbial nodes and soil properties.

- To evaluate the effect of the cultivation systems on potential microbial community related to predictive microbial general pathways, especially for the nitrogen cycle genes.

State of the art

1. The importance of Agriculture for population and the role of soil in Agriculture

One of the most significant changes in the history of mankind has been the agriculture. Agriculture is a keystone to feed the world population which has been increasing over the years (1). The model of agriculture carried out since the green revolution is based on the intensive use of chemical fertilizers, herbicides, pesticides and monocultures (Figure 1), which have caused the loss of wild land; loss of water due to the high amount needed by crops; loss of soil fertility; high contamination due the fertilizer inputs affecting soil and water; high energy consumption mainly from non-renewable systems and a high loss of diversity due wide arable land extension (2). For these reasons, the expansion of agriculture in this way has involved the humanity's largest impact on the environment. However, the big concern of global population increasing in 81 million people per year by 2050 (3), which will require 70% of increase in crop production (4); and the reduction of soil availability for cultivation and less productive each day needs a revolution to revert the situation.

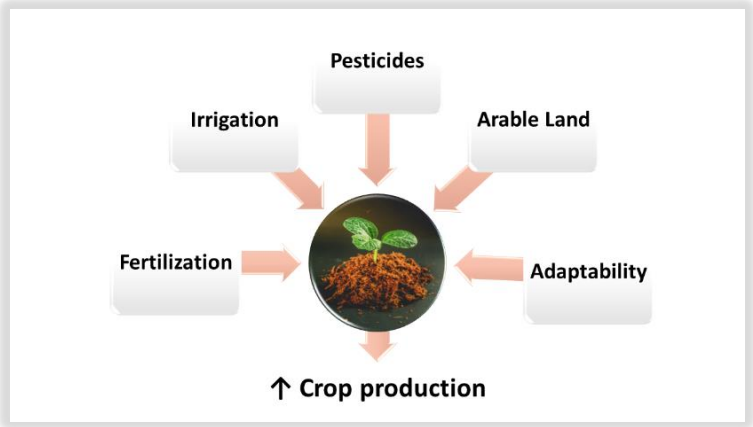


Figure 1. *Radial scheme showing the basis of green revolution to increase crop production.*

One of the most important and less studied resources in agriculture is the soil. The soil can be defined according to Soil Science Society of America (SSSA) as "the unconsolidated mineral or organic material on the immediate surface of the earth that serves as a natural medium for the growth of land plants", however, although soil is a wide resource; it is finite and it cannot be replaced, at least, in the geological human time (5). The soil is made up of organic matter composed by plants, animals, and microorganisms (that are alive, dead or in some stage of decomposition); inorganic material (silt, sand, and clay particles), water and gases. Between them, an exchange of molecules is produced through physical, chemical, and biological processes. Organic matter increases soil aggregation and structure, the soil water holding capacity, and some other important functions such as movement of nutrients to the microorganisms and plants. Microorganisms and their activity play and important role in the conservation, maintenance, and recovery of the soil when it is subjected to degradation processes (6).

The soil microorganisms mediate in the biogeochemical cycling for availability of soil mineral nutrients such as nitrogen, phosphorus, and sulphur, which are the major growth promoting nutrients to the plants and necessary to stablish a natural soil fertility. In this respect, it is more precise and important to talk about soil health; it could be defined as "the continued capacity of soil to function as a vital living system, within ecosystem and land-use boundaries, to sustain biological productivity, promote the quality of air and water environments, and maintain plant, animal, and human health" (7). As it has been mentioned above, the soil is affected by intensive agricultural practices such as use of pesticides, herbicides, and inorganic fertilizers, that produce soil degradation. This includes alterations such as: erosion, salinity, loss of organic matter, fertility decline, acidity or alkalinity, loss of structure and soil contamination (toxic chemicals and pollutants). According to Panagos (8), this soil degradation is expected to continue and increase in the coming years. The evolution of soil degradation in Europe and United Kingdom (UK) in 2050 using three Representative Concentration Pathway (RCP) RCP 2.6, RCP 4.5, and RCP 8.5 is represented in the Figure 2. This model shows an increase

in soil erosion by 10-50% for RCP 2.6 scenario if carbon dioxide (CO_2) emissions start declining by 2020 and go to zero by 2100 (Figure 2B); for the RCP8.5 scenario, where emissions continue to rise throughout the 21st century (Figure 2D), the soil erosion would be increased a mean range of 20-100%. Therefore, depending on the level of human activity, there may be a condition of erosion of the soil or another, highlighting the effect of anthropogenic activity. The loss of organic matter with higher CO_2 emissions, the loss of fertility and productivity and the use of fertilizers and pesticides in agriculture soils have dramatically increased in the last decades and although fertilizers and pesticides are very useful to crop growth and crops protection against insects, pathogens, and weeds among other pests; they also compromise the ecosystem and environment due its overuse and environmental consequences. Currently, around 2 million tonnes of these products are utilized worldwide polluting soil, air, and water (9).

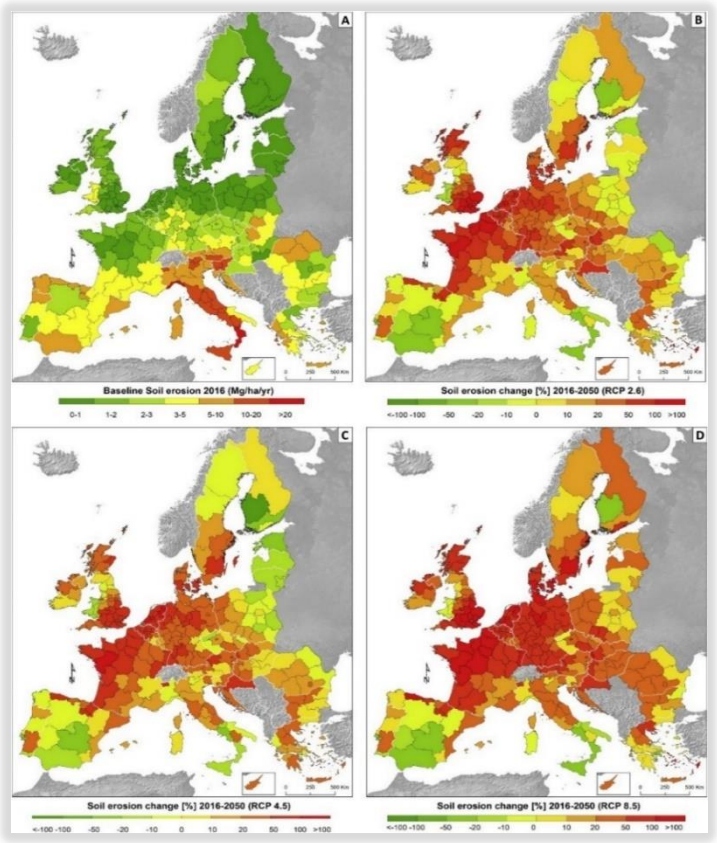


***Figure 2**. Soil erosion across Europe in 2016 (A) soil erosion change between 2016-2050 based on different Representative Concentration Pathway (RCP) scenarios (B) RCP 2.6, (C) RCP 4.5 and (D) RCP 8.5 (9).*

Prăvălie (10) concluded that 25% of European land was identified with high or very high risk of desertification because of the high erosion rates, low levels of soil organic matter, and high abundance of shallow soils and salinisation problems. These facts joined by the unique characteristics of the Mediterranean-climate regions characterized by hot dry summers and cold wet winters, and that currently is threatened by increases of drought and high temperature events associated with climate change (11), lead to degradation and

desertification of agricultural soils, as happens in Murcia Region.

Panagos (12) showed that soil deterioration of around 12 million of hectares of agricultural areas will have an annual loose around 0.43% of productivity, resulting in loss of €1.25 million. In the year 2020, agriculture was the third contributor of gas emission (14%), preceded by the industry (20.8%) and the transport (27.0%); where dioxide carbon was the most abundant gas (77.7%) followed by methane (13.7%) and nitrous oxide (6.6%) (13).

2. Sustainable agriculture for population

Sustainable agriculture implies friendly environmental methods to farmland, which allows to produce food, and at the same time soil and environment conservation. European Union is more precise and through the Common Agricultural Policy (CAP) promotes an economically and environmentally sustainable agriculture (14). There are many kinds of managements that it can be used to change from conventional to sustainable farming, such as reducing tillage, increasing crop rotation or intercropping, use of organic amendments as compost or manure, reducing the use of chemical fertilizers and replacing the chemical fertilizer by an organic fertilizer, furthermore, making an efficiency use of water and soil nutrients.

2.1. The use of organic amendments and organic fertilizers for a sustainable agriculture

Organic amendments provide macro and micronutrients, including carbon, for the restoration of soil physical and chemical properties (15); their use allows better management of often-finite resources. Organic amendments are composed by plants and animals that are alive, dead or in some stage of decomposition (16); its composition depends on material they are composed so there is a wide variety of amendments for example manure, compost, sludge, biochar, slurry, etc. (16). In general, they are a compound of fresh and stabilized organic materials. The fresh organic materials have higher nutrient content, but its management must be also done with care due to unbalanced composition, that could contribute to the alteration of biochemical flows of nitrogen (N) and phosphorus (P) (17), and the potential incorporation of human pathogens (18). The stabilized organic materials have been matured through process such as composting, that permits to manage them in a safer way, without pathogens and weeds; they incorporate less amount of nutrients, but their use can improve soil structure by increased water and oxygen permeability; it also helps to resist compaction and increases soil binding properties, which reduces erosion. The choose one organic amendment or another usually is a farmer's decision, with the guidance and help of the public authorities (14).

Organic fertilizers are of natural origin and contain moderate amount of plant essential nutrients (19). They gradually release nutrients into the soil solution and maintain nutrient balance for healthy growth of crop plants; furthermore, they also act as an effective energy source of soil microbes which in turn improve soil structure and crop growth; they are safer alternatives to chemical fertilizers (20).

2.2. Intercropping and crop rotation for a sustainable agriculture

Intercropping is a particular type of crop rotation where it uses spatial diversification (alternate crops mixed in the available area), with the capability to have different rooting ability, canopy structure, height, and nutrient requirements (21,22) and the goal to maximize the utilization of the growth resources available on the land. **Crop rotation** can work a little differently, depending on the crop type and how the land is managed. A single crop type is growth at once, but it is combined with other crops during the whole season (21,22). Crops that need large amounts of nitrogen would be cropped after crops, like legumes, which add nitrogen back into the soil. They potentiality improve soil condition and boost system productivity (23).

Both cases have **advantages** but also **disadvantages.** Briefly, they can effectively improve the climate resilience of crops through the enhancement of water dynamics, soil health and biological conditions in planting systems (24,25); They can also increase the structure of microbial communities as well as soil stability and yield production (26); reduce the attack of insects and pathogens and help to disease control and thus reducing the use of pesticides/fungicides (27). However, some disadvantages must consider for both cultivation systems, especially the space loss since the piece of land with crop rotation will not always be able to be cultivated with the main crop. In the intercropping system, the kind of mixed crops must also be taken into account, otherwise the crops will compete for light, soil nutrients and water, furthermore, the growth phases of each crop has also to be considered, due to their requirements will change (28).

2.3. Reduction of crop tillage

Reduced tillage is characterized by reduced practices such as ploughing, harrowing, and all the tillage operations ordinarily applied to prepare the soil for seed germination, seedling establishment and crop growth and production (29). Reduced tillage or no tillage helps to improve soil properties, preserves, and increases soil organic matter, and hence reduce soil erosion. Moreover, reduce energy consumption by agriculture machinery and enhance soil drainage (29). Indeed, several ecosystem services are provided by the minimum soil disturbance, including water regulation, carbon storage, soil stability, protection of surface soils from erosion, enhanced water infiltration, increased soil fertility through enhanced nitrogen stocks (in the long term), improved soil, water, and air quality, reduction of soil erosion and fuel consumption (30). All these elements are of the highest importance to reduce the vulnerability of the agricultural systems; they increase their adaptation capacity to climate change, contributing also to the mitigation objectives.

3. The importance of the soil microbiota and its relationship with the soil biogeochemical cycles for enhancing a sustainable agriculture

Several physiological processes in the soil, including photosynthesis, respiration, or nitrogen fixation between others, are regulated by major biogeochemical reactions carried by microorganisms that intimately link to the soil biogeochemical cycles of carbon, nitrogen, phosphorus, and sulphur (31). Microorganisms, plants, and soil interact through different processes. The soil provides nutrients to both microorganisms and plants, the microorganisms obtain these nutrients, but at the same time they change soil properties through their activity; they have also an important role in soil stabilization through soil aggregation (32). Furthermore, they can also change plant function through direct process such a manipulation of hormone signalling or soil-borne pathogen protection (33); or provide available nutrients to the plant by their mineralization, promoting plant growth through plant-soil feedbacks (34); at the same time, they also use root exudates as carbon source (31).

Microorganisms can be classified into two simple wide groups: autotrophs and heterotrophs. The first group are known as producers because they are capable to make their own food from raw materials and energy; the second group, on the contrary, are known as consumers because they consume to the producers or other consumers. Plants depend on the activity of heterotrophic soil organisms since microorganisms are capable to degrade organic matter that supplies to the plants (35). In this context, the soil is defined as the largest reservoir of carbon, but it is limited to be available to heterotrophic microorganisms (17,35), mainly due to low concentration of biodegradable organic matter, being predominant the recalcitrant carbon sources; the low C:N ratio and the physicochemical protection of organic matter within the soil mineral matrix (32). The carbon limitation availability for microorganisms that cannot obtain energy, could cause the limitation of other nutrients, such nitrogen (17) or phosphorus (36) limiting plant growth.

3.1. Involvement of microorganisms on carbon cycle

Carbon is the main element of the biological systems that constitutes more than 40% of the living organisms on dry matter basis (37). Carbon cycle is regulated by the equilibrium between photosynthesis and respiration; it is intimately coupled with the cycles of the other macro and microelements required for the microbial metabolism (38). The fixed carbon from soil is reverted to the atmosphere through mineralization of organic matter. Restoration of CO_2 takes place by respiration in the living organisms. CO_2 is the main respiratory flux in the well-aerated soils, whereas, in the anoxic soils, CO_2 is reduced to methane (methanogenesis) (39). Terrestrial organic carbon compounds are decomposed by soil-inhabited microorganisms that are mainly formed by fungi and bacteria.

Mycorrhizal fungi play an important role in soil carbon cycling by obtaining carbon directly from the host plant as obligate symbionts (arbuscular mycorrhizal fungi; AMF), or by mineralizing organic carbon as facultative symbionts (ectomycorrhizal fungi; ECM) (40). **Cellulose decomposition:** cellulose is the major abundant structural component of plants' primary cell wall that is decomposed by different fungal and bacterial species (41); **hemicellulose and lignin decomposition:** hemicellulose is the second most abundant biopolymer in plant cell walls after cellulose; it is composed of different pentose and hexose monosaccharides and uranic acid; it is decomposed by soil microbes through extracellular enzyme hemicellulose. Lignin is the third most abundant component of plant cell walls, providing rigidity to the plants, and thereby, extremely resilient to the microbial degradation (42).

3.2. Involvement of microorganisms on nitrogen cycle

Nitrogen cycle is one of the primary biogeochemical cycles in which different microorganisms are involved in **nitrogen fixation, nitrification, denitrification,** and others.

Nitrogen fixation is a well-known process in which atmospheric nitrogen (N_2) is converted into organic nitrogen through ammonia production that can be readily metabolized by most of the living organisms. This process is extraordinary and crucial to increase nitrogen soil storage, in fact, it is tried to synthetically recreate it (43); therefore, increasing the number of nitrogen fixation in the soil can be a challenged on our century (44,45). **Nitrogen fixation** is mainly executed by free-living, associative, and symbiotic bacteria (46). **Nitrogen assimilation** is the utilization of previously fixed nitrogen which can be carried out by plants, bacteria and some fungi through different enzyme activities; it is a fundamental step for obtain nitrogen compounds such as amino acids. On the other hand, **ammonification** is also an important pathway which allows recover NH_4^+ from some complex compounds with amino or amide groups; they can also transformed through **nitrification**, which gets nitrates and nitrites from ammonium increasing the storage of these two inorganic nitrogen compounds. The microbes gain energy by oxidizing ammonia and use CO_2 as the carbon source. Chemolithoautotrophic bacterial groups, Ammonia Oxidizing Bacteria (AOB), and Nitrite Oxidizing Bacteria (NOB) carry out the process of nitrification. Several heterotrophic microorganisms also contribute to this process. **Denitrification** reduces the availability of nitrogen in soils because denitrification pathway conversion of the NO_3^- to the gaseous N_2, involving NO_2^- production as an intermediate step. Anammox bacteria mediate the conversion of NH_3 and NO_2 into gaseous N_2 under anaerobic conditions (47). In this sense, a greater number of soil microorganisms capable of capturing and transforming atmospheric nitrogen could be useful for current agriculture, because it would prevent the fertilizer inputs and the soil balance would not be damaged.

3.3. Involvement of microorganisms on Phosphorus cycle

Phosphate is also one of the fundamental nutrients for plant growth, but also one of the most limiting that must be added to soils. For that, microorganisms take, at this point, a fundamental role to increase the amount of phosphate available for crops. These kinds of microorganisms are known as Phosphate Solubilizing Microorganisms (PSMs) which can release phosphate enzymes as well as organic acids, reducing soil pH, and increasing chelation activities with additional P adsorption sites (48); PSMs can dissolve the soil P into available forms by plants mainly acid phosphoric (PO_4^{3-}), hydrogen phosphate (HPO_4^{2-}), and dihydrogen phosphate ($H_2PO_4^-$) (49). The study of the contributions of PSMs to soils and plant nutrition could help to understand and know the requirements of these microorganisms and work with them; it could be able restore the soil phosphorus avoiding extracting it from non-renewable resources (48,50).

4. Indicators of soil quality for evaluating sustainable agriculture practices

Agricultural soils have traditionally been managed mainly for productivity through food, feed, fibber, and timber production. They sustain a wide range of functions or processes, related to environmental resilience (51). Soil quality comprises inherent soil quality (e.g., climate, organisms, topography, parent material and time); which refers to those aspects that change because of land use and soil management (e.g., physical, chemical, and biological parameters and soil microbiota) (52). Intensive agricultural management has been highly successful in increasing production, but often with detrimental effects on soil properties. These impacts can, in turn, disrupt soil processes; and soil ecosystem services, defined as the benefits for humankind derived from ecosystems (53). In this context, the assessment and the monitoring of soil quality are affected by agricultural management. Redesigning agricultural systems (54) towards increasing both agricultural productivity and maintenance of soil quality is paramount.

Soil biota has a primary role in many soil processes, and they are more easily and quickly influenced by different perturbation, in addition, they are closely linked with physical and chemical properties (37,42,44). For this reason, the composite use of chemical, physical and biological properties is crucial to effectively assess soil quality. Briefly, according to Brünemann (55), the most used **physical parameters** are, among others, the bulk density, that allows us to evaluate the resistance of the soil to the growth roots; soil texture, structure and soil aggregates, water holding capacity, water storage, soil depth and porosity. Belong to **chemical indicators,** some of the most used are total organic carbon (TOC), electrical conductivity (EC), total nitrogen (TN), phosphorus (P), potassium (K) and other macronutrients such as Mg, S and Ca available for the plants (56,57).

Regarding to **biological indicators** such as, microbial biomass carbon or N (58) and especially the metabolic quotient (qCO_2), that have been the most used (59). Soil microbial activity has been deeply used as soil biomarkers through the measure of enzyme activities, that give a potential general microbial activity (Dehydrogenase) and related to different biogeochemical cycles (C, N and P), such as β-glucosidase, urease, or phosphatase respectively (60,61). These measurements together with **molecular techniques** can provide information about microbial processes and plant-microbe interactions in soil (62).

5. Tools to evaluate soil microbial community structure and functionality

Approaches have been developed to study the structure, diversity, and activity of soil microbes to better understand the biology and plant-microbe interactions in soils. A better knowledge of the microbial community is important to improve our understanding of the soil ecosystem, but it is also a challenging endeavour, due to the difficulties of cultivating or directly observing some of the soil microorganisms, consequently, many of these microbial communities are not yet well characterized (63). Bacterial and fungal populations have been frequently studied using **biochemical** and **molecular** based methods that have advantages and disadvantages.

Between **biochemical methods** the most used are *Community Level Physiological Profile* (CLPP), based on sole carbon substrate utilization profiles; it is a rapid screening method used to differentiate between microbial communities, but it only works with culturable microorganisms, therefore, information about uncultivable microorganisms (which corresponds to 99%) is missed (64); *Fatty Acid Methyl Ester analysis* (FAME) provides information of microbial community based on grouping fatty acids (65), because they have a constant proportion depending on the microbial community, however, this method has limitations, especially for fungal community, since it needs around 130 to 150 spores and could obscure detection of minor species (66). Furthermore, the fatty acids are sensitive to environmental factors such temperature, nutrition etc., and it could alter the *FAME* profiles (66), however, this sensitivity can be overcome by using *Phospholipid Fatty Acid* (PLFA) that are more dependent on microbial community rather than on environmental changes (67).

Culture-independent **molecular methods** used in studying soil microbial biomass, diversity, and activity include analyses of selected genes such as 16S rRNA for bacteria or 18S/ITS rRNA for fungi through Polymerase Chain Reaction (PCR)-analysis of extracted Deoxyribonucleic acid (DNA)/Ribonucleic acid (RNA) and from the whole genome (68) that show different advantages and disadvantages (Table 1).

Guanine to cytosine (G+C) uses the differences in the G+C to study the bacterial diversity of soil microbial communities (69); they differ in their G+C content and taxonomically related groups only differ between 3-5% (69). This technique can quantify (as well rare members of the community) and it is not influence by PCR biases (Table 1). *Nucleic acid reassociation and hybridization* where diversity can be tested by DNA reassociations, with higher complex samples DNA reassociates will decrease (70).

Table 1. *Advantages and disadvantages of molecular methods to study microbial community (64,70).*

METHOD	ADVANTAGES	DISADVANTAGES
Guanine plus cytosine (G+C)	✓ Not influenced by PCR biases ✓ Quantitative ✓ Includes all DNA extracted and rare members of community	⊗ Large quantities of DNA are needed ⊗ Dependent on lysing and extraction efficiency ⊗ Low resolution level
Nucleic acid reassociation and hybridization	✓ Not influenced by PCR biases ✓ Total DNA extracted ✓ Study of DNA or RNA	⊗ Lack of sensitivity ⊗ Hugh copy number of sequences is needed
Terminal restriction fragment length polymorphism (T-RFLP)	✓ Simpler banding patterns than RFLP ✓ Can be automated, large number of treated samples ✓ Highly reproducible ✓ Compare differences in microbial communities	⊗ PCR biases ⊗ Type of Taq can increase variability ⊗ Choice of restriction enzymes will influence community fingerprint ⊗ Dependent on lysing and extraction efficiency ⊗ Choice of universal primers
Ribosomal Intergenic Spacer Analysis (RISA)/Automated Ribosomal Intergenic Spacer Analysis (ARISA)	✓ Highly reproducible community profiles	⊗ Requires large quantities of DNA ⊗ Resolution tends to be low-PCR biases
Single Strand Conformation Polymorphism (SSCP)	✓ Same as DGGE/TGGE	⊗ PCR biases ⊗ Some ssDNA can form more than one stable conformation
Random Amplified Polymorphic DNA (RAPD)	✓ Quick and easy to assay ✓ Low quantities of template DNA	⊗ Low reproducibility ⊗ Highly standardized experimental procedures because of their sensitivity to the reaction conditions.
Denaturing and temperature Gradient Gel Electrophoresis (DGGE and TGGE)	✓ High number of samples can be analyzed simultaneously ✓ Reliable, reproducible and rapid	⊗ PCR biases ⊗ Dependent on lysing and extraction efficiency ⊗ Handling sample greatly influences the results ⊗ Study of co-migration and dominant species
Amplified Ribosomal DNA Restriction Analysis (ARDRA) or Restriction Fragment Length Polymorphism (RFLP)	✓ Detect structural changes in microbial community	⊗ PCR biases ⊗ Type of Taq can increase variability ⊗ Choice of restriction enzymes will influence community fingerprint
DNA microarrays and DNA hybridization	✓ Some as nucleic acid hybridization ✓ Thousands of genes can be analyzed ✓ The use of genes or DNA fragments increased specificity	⊗ Only detects the most abundant species and culturable microorganisms ⊗ Low diversity system has higher accurate
Shotgun metagenomic sequencing	✓ High taxonomic resolution ✓ Functional profiling ✓ Including viruses	⊗ Expensive ⊗ Complex bioinformatic pipelines
Amplicon-based Next Generation Sequencing (NGS)	✓ Culture-independent ✓ Extensive and in-depth information	⊗ Results are relatively rather than quantitative ⊗ Does not determine cause-and-effect relationships. ⊗ rRNA sequencing can be biased
Quantitative-PCR (QPCR) and reverse transcription (RT-QPCR)	✓ Robust, highly reproducible, sensitive, and fast ✓ Quantitative	⊗ Only for targeting of known genes ⊗ Expensive equipment ⊗ The multiplexing is limited

The generation of amplified fragments by selected primers although are likely to underestimate diversity can be used to evaluate the community structure of microbial populations tracking the dominant members of the community (71). Genetic fingerprinting techniques based on PCR analysis can be divided into two groups according to the differential electrophoretic migration on agarose or polyacrylamide gels: **1**. the migration depending on the size of the sequence that include: *Terminal restriction Fragment Length Polymorphism* (T-RFLP), R*ibosomal Intergenic Space Analysis*

(RISA)/*Automated Ribosomal Intergenic Spacer Analysis* (ARISA), *Single Strand Conformation Polymorphism* (SSCP) and *Random Amplified Polymorphic* DNA (RAPD); **2.** the migration depending on the sequence, *Denaturing Gradient Gel Electrophoresis* (DGGE) and *Temperature Gradient Gel Electrophoresis* (TGGE), *Amplified Ribosomal* DNA *Restriction Analysis* (ARDRA) or *Restriction Fragment Length Polymorphism* (RFLP). In general, all these techniques stand out for their reproducibility and the choice could be dependent of samples to analysed, the technical capacity and the available resources (Table 1). Another PCR based method are *DNA microarrays* where PCR products amplified from total DNA directly hybridized to known molecular probes which are attached on the microarray (72). In general, the hybridization signal intensity is directly proportional to the abundance of the microorganism (Table 1). *Quantitative PCR* (qPCR) can measure the abundance and expression of taxonomic and functional gene markers (76).

On the other hand, one of the techniques that is gaining ground is shotgun metagenomics, an untargeted ('shotgun') sequencing of all ('meta-') microbial genomes ('genomics'), capable to profile taxonomic composition and functional potential microbial community (73), however, although it has a very high resolution identifying at species level, it continues being a very expensive method and large computing resources so important bioinformatics skills are required, which makes it currently difficult to access (Table 1) (74), thus, one of the most common techniques used for this purpose is the *Amplicon-based Nex Generation Sequencing* (NGS) that allows the study of non-cultivable microorganisms, it is fast and requires less resources than the shotgun (75).

5.1. High Throughput Sequencing/Next Generation Sequencing

High Throughput Sequencing (HTS) technologies also known as Next Generation Sequencing have widely used to analyse the genetic and functional diversity of microbial communities in soils and rhizosphere (77). The HTS technologies are capable of sequencing hundreds of thousands of sequences, sometimes up to a million, at once, that produce huge volume of sequencing data. For this reason, it has needed to develop algorithms which can handle large amount of data.

It has rapidly evolved over the past 15 years and new methods are continually being commercialized. The term "next generation" has implied a next step in the development of DNA sequencing technology and suggests that there will be a "next-next" generation naming of new technologies in the future. Due to advances in nanotechnology and bioinformatics, alternative technologies have been created to increase the throughput of DNA and RNA sequencing have emerged. The most widely used platforms for massive parallel sequencing, for assessing soil microbial diversity, are **Roche 454 sequencing** (Roche Molecular System Inc., Meylan, FR), **Illumina Technology** (Illumina Inc., San Diego, CA, USA), and **Ion Torrent technology** (PGM, London, UK).

Based on the PCR technique, thousands of copies from one gen can be obtained through these platforms. The sequencing can be divided into three general steps a) library preparation, b) amplification, and c) sequencing (78). In general terms, the **library preparation** is the first step of the NGS; the DNA or RNA samples are prepared to be compatible with the sequencer, it is commonly created by a fragmenting DNA and adding specialized adapters which will depend on the kit and the platform used in the study, such an Illumina or Ion Torrent. One of the biggest differences between both platforms are the amplification and sequencing, first, **Illumina sequencing technology** is based on technology known as "bridge amplification" wherein molecules of DNA (around 500 bp), with the correct adapters ligated on each end are used as substrates. Then, a complementary string to the ligated adapter is on a solid support, and when both complementary adapters are joined, a complementary chain begins to be created giving rise to sequences known as: forward and reverse (79). During synthesis reactions, each nucleotide incorporates its own fluorescence; it is detected to identify each nucleotide (78,79). On the other hand, **Ion Torrent technology** converts nucleotide sequence into digital information; DNA fragments (200-1500 bp) are ligated to the adapters, then they will join to the complementary chain on the beads, where it will be amplified in this system. When a correct nucleotide is incorporated across from its complementary base, a hydrogen ion is released; it produces a slightly pH of the solution that can be recorded as a voltage change by ion sensor (78,79).

To perform the **amplification,** a well-known and informative gene such as 16S rRNA for bacteria and the 18S rRNA/ITS for fungi is used, which common regions presenting polymorphisms in some nucleotides that allow to classify the microorganisms into different taxonomy. 16S rRNA has a total of nine hypervariable regions which allow to study bacterial diversity, but the most common hypervariable regions used is the number four, that can be amplified with the primers 515F/806R (80). There is no consensus on the most suitable region and the final election belongs to the researcher (81); however, this is changing, and some tests are appearing in which they use more than one hypervariable region that can provide more taxonomic information than sequencing only one single region (82). For fungal communities, the target region for amplification most common is ITS1F and ITS2 and the usual primers used are generated followed the Smith and Peay (83) methodology based on White et al. (84).

The **analysis of sequences** obtained can be stored by sequencing platform in different file formats, although the most common one is **FASTQ**. FASTQ is a plain text file full of thousands of lines which contains a) header, b) sequence (with the adapter, barcode, and primer), and c) the quality line which corresponds to phred score with American Standard Code for Information Interchange (ASCII) characters; it encodes the base-calling error probabilities. The quality line allows us to remove sequences that are considered unreliable. The adapters sequences are short oligonucleotides used to be ligated to the ends of DNA fragments of interest which can be combined with the primers for amplification; the primer is a short, single-stranded DNA sequence used in the PCR which is used for the polymerase to amplify the target region; the barcode is a known nucleotide

small sequence (usually between 6 and 10 nucleotides) that allows us to identify and split all the sequences of each sample, as well-known as multiplexed sequencing analysis, increasing lab efficiency and reducing sequencing costs per sample.

The first step to obtain the sequences is to remove the adapter, primer, and the barcode from the FASTQ through bioinformatic programs such as ***cutadapt*** (85); although some platforms such as Ion Torrent incorporate its own software to remove and denoising it, apart from that, there are programs than can help to remove the errors from sequencer, such ***ACACIA*** (86). Nowadays, there are multiple open access platforms that can be used for sequence analysis (bioinformatic analysis). The most common ones used are ***Quantitative Insights into Microbial Ecology II* (QIIME2)** (87) and sequence analysis suite for research on microbiota such as ***Mothur*** (88). These tools allow to process all the files from the FASTQ file (considerer as raw data) until the depurated and classified sequences are obtained.

The Figure 3 shows a general overview from ***QIIME2*** showing various possible workflows for examining amplicon sequence data.

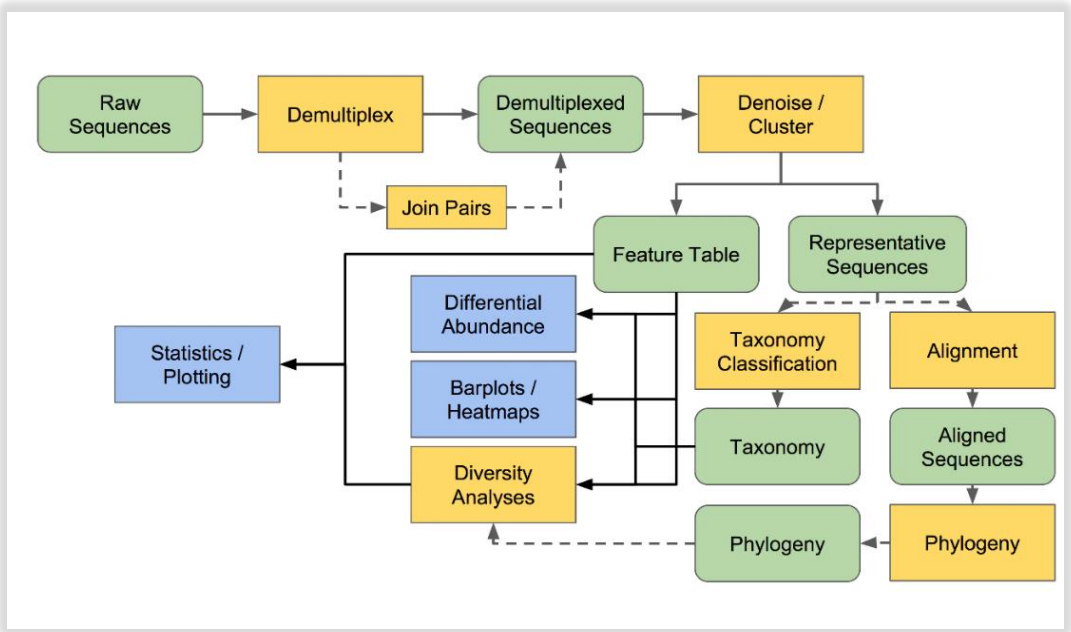


Figure 3. *Complete pipeline for 16S amplicon sequences analysis using QIIME2 from (87). Raw data are entered into QIIME, forward and reverse sequences are joining, demultiplexing and denoising, then taxonomic classification and statistical analysis.*

Briefly, it includes the removal of the adapters, barcodes, primers and separate the sequences by samples e.g., using ***cutadapt***, then, those files must be imported into ***QIIME2***, and the analysis continues with a denoise bioinformatic process; they allow us to remove incorrect sequences from sequencer. This process is commonly carried out by ***dada2*** (89) or ***deblur*** (90). In the next step, traditionally, the sequences have been grouped into cluster making Operational Taxonomic Unit (OTU) before taxonomic classification

with the aim to reduce the noise of one or two misassignment nucleotides from PCR; other way of remove the noise is by Amplicon Sequence Variant (ASV). The difference between ASV and OTU is the way they are generated. ASV through precision algorithms such a ***dada2***, errors will be removed, and the resultant sequences are clustered, assuring that each ASV corresponds to one taxonomy group; in the other hand, OTUs are clusters of grouped sequences with a determined similarity (commonly 97%).

Once errors have been reduced or eliminated from samples the workflow continues with the taxonomic classification (Figure 3); it implies to compare the data from the experiment against a depurated database being the most used ***Silva*** (91) or ***Unite*** (92) for bacterial and fungal sequences respectively. It should be recalled that there are other kinds of databases available for users such a ***Greengenes*** (only for 16S) (93), although this database is not updated since 2012-2013, the database from ***National Centre for Biotechnology Information*** **(NCBI)**, or ***GenBank*** (94) that is not as refined as ***Silva*** or ***Unite***. In addition, it is important to note that currently it is important to use repositories to upload the sequences, and that they are accessible to everyone, such a ***Sequence Read Archive*** **(SRA)**, ***MG-RAST or European Nucleotide Archive*** **(ENA)**. When the taxonomic classification is obtained, then begin the final step: the statistical analysis (Figure 3), in which is usually to study the taxonomies, the diversity; the graphic representations also are performed.

5.1.1. Functional gene prediction analysis

Soil microorganism are important, but also their functionality. The functional meta-genomic approaches allow the identification of genes encoding functions of interest in the soil microbiological processes e.g., carbon, nitrogen, contamination by metals etc. Computational prediction tools such as ***Functional Annotation of Prokaryotic Taxa*** **(FAPROTAX)** (95), ***Phylogenetic Investigation of Communities by Reconstruction of Unobserved States*** (**PICRUSt**) (96), and ***Tax4fun*** (97) offer the possibility to translate structural community data into ecosystem functions in a cost-effective way, that use the link between bacterial 16S rRNA gene amplicon sequencing and functional gene annotations of prokaryotic reference genomes (98). Briefly, ***FAPROTAX*** is a database that maps prokaryotic clades (genera or species) and using the current literature on cultured strains established metabolic or other functions (99). On the other hand, ***PICRUSt*** and ***Tax4fun*** work in a similar way, using **Greengenes** and ***Silva*** as reference database respectively. In general, ***PICRUSt2*** seems to get a better result in functional pathways (100) whereas ***Tax4fun2*** is more accurate on more specialized methane metabolism pathways (100,101).

6. Statistical analysis

Bioinformatics is an interdisciplinary field of research to analyse big datasets from HTS technologies, which is fed on disciplinary knowledge of not only Biology and Computer Science, but also Statistics (102). Indeed, NGS data are continuously posing new challenging issues on computational statistics and statistical computing whose developments are being implemented in bioinformatic platforms (QIIME, Mothur, etc.) and R/Bioconductor packages using the R statistical programming language (103). Thus, novel contributions and improvements of standard statistical techniques such as Permutational Multivariate Analysis of Variance (PERMANOVA), Analysis of Similarities (ANOSIM), Principal Coordinates Analysis (PCoA), Co-ocurrence network analysis, Redundancy Analysis (RDA), Non-Metric Multidimensional Scaling (NMDS), Linear Discriminant Analysis (LDA) can be performed by ***libraries*** designed for sequencing-based microbial community data analysis such as the ***vegan*** package (104) and even by open-access bioinformatics pipelines like ***Molecular Ecological Network Analysis*** (**MENA**) (105) and ***Galaxy Linear discriminant analysis Effect Size*** (**LEfSe**) (106), and also more general software as ***igraph*** (107) or the open source ***Cytoscape*** software (108).

6.1. Statistical Methods for analysing data from laboratory-prepared soil samples

Soil properties are commonly analysed in agricultural research studies by using Statistics (109). In general, the statistical techniques are usually classified into a) ***univariate methods,*** focused on the analysis of the variables one by one, and b) ***multivariate methods***, aimed at the study of two or more variables jointly.

By ***univariate analysis techniques,*** normality and homoscedasticity conditions are usually tested a priori, to ensure reliable results derived from parametric techniques such as Analysis of Variance (ANOVA), which are followed by multiple comparison tests to determine significant differences, when the hypothesis of equality of means is rejected. However, if normality and homoscedasticity are not met, different alternative procedures to approximate data to them might be found in the literature, such as the application of transformations on the variables of interest (110) or implementation of resampling methods (111). Otherwise, nonparametric tests are carried out to identify informative variables, since such statistical procedures are robust to deviations from normality and homoscedasticity (112).

Using ***bivariate analysis techniques***, interrelationships between different variables can be evaluated through parametric correlations by ***Pearson correlation coefficient*** or non-parametric correlations by ***Spearman rank correlation*** coefficient or ***simple linear regression*** analysis.

More general, ***multivariate statistical methods*** might be classified into three different categories (113,114): a) **exploratory methods**, b) **interpretive methods,** and c) **discriminatory methods**.

The **exploratory methods** can be used to explore the relationships among observations from the values of variables measured on the sample, among which Principal Component Analysis (PCA) (115), CA, NMDS, HCA and k-means analysis (116) are some of the most applied.

The **interpretive methods** are composed by constrained techniques which consider explanatory variables in addition to measured variables with the aim of finding axes maximizing the association between both groups of variables, as for instance Canonical Correspondence Analysis (CCA), Canonical Correlation Analysis (CCorA) (117), Generalized Linear Model (GLM) and MANOVA. In a more general framework, Co-inertia Analysis (CIA), Procrustes analysis (PA) (118), RDA, db-RDA, Principal Response Curve (PRC) (119), ANOSIM and PERMANOVA can be also included in such a category.

Finally, **discriminatory methods,** that encompass an extension of the interpretive multivariate techniques called discriminant analysis (DA) used for computing discriminant functions or hyperspace functions which maximize the separation among different groups of observations, such as applying LDA, Discriminant Function Analysis (DFA) (120), Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA), Support Vector Machine (SVM) and Random Forest (RF) (121).

6.2. Statistical Methods for analysing data from high-throughput technology

By nature, the sequencing data are multivariate, so analysing them not only increases the execution time and computing capacities because of the multivariate statistics required, but also the complexity of the interpretation since a great proportion of soil organisms are not yet characterized in taxonomic and functional term (122). Before the statistical data analysis is usual to evaluate the coverage obtained from sequencing mainly through ***rarefaction curves*** (frequently taken to the minimum number of reads of all sample) that plot the number of species (OTUs or ASVs) against the number of reads per sample. This plot allows to easily visualize if the sequencing reaches sufficient depth to analyse the full diversity of the sampled microbial community. Although there is no consensus about whether to rarefy or not to rarefy, it is one of the most used methods (123,124).

The distribution of these microbial species in the soil is important, because in general, a higher diversity is related to a higher capacity to maintenance soil resilience (125). There are three different diversity indices: a) ***α-diversity*** which is defined as the mean diversity of species in different sites within a local scale, b) ***β-diversity*** which is the ratio between regional and local species diversity and c) ***γ-diversity***, which is the measure of the overall diversity for the different ecosystems within a region. The most used indices are ***α-diversity*** and ***β-diversity*** (126). To study the ***α-diversity*** in a microbial community, different indices can be used such a Shannon (diversity) (127) or Chao (richness) (128), and univariate techniques such as ANOVA or its counterpart nonparametric Kruskal-Wallis test procedures are applied. Whereas specific multivariate analysis such as PERMANOVA or ANOSIM, using dissimilarity matrices (generally Bray-Curtis), are used for ***β-diversity***, that are usually represented through ordination explorative methods such as PCoA.

One of the most common approaches from amplicon data is the study of taxonomic classification, generally at phylum, family, and genus level. For that purpose, the different taxa are split from the featured table obtaining abundance or relative abundance for each classification. Similar procedure is carried out on the data from ***PICRUSt***, with the difference that classification corresponds to potential gene prediction instead of taxa classification. To test the differences between the classifications the use of techniques such ANOVA (when data fulfilling the assumptions), Kruskal-Wallis test (when normality does not fulfil) or Welch's test (when homoscedasticity is not met) are used with the aim of finding those microorganisms or predicted genes that are clearly different between different managements or treatments.

Specific algorithms have been developed for this kind of data, such as **LEfSe** (129), which allows to know the contribution of each taxon which is described as biomarker. Figure 4 shows the different steps of LEfSe algorithm. First, the required experimental analysis is performed on dataset which can come from 16S, Whole Genome Sequencing (WGS) or mRNA; then, the corresponding bioinformatic pipeline will be carried out to get the featured table (containing taxa or potential gene prediction). At this point, the algorithm performs a nonparametric Kruskal-Wallis sum-rank test (130) to detect features with significant differential abundance respect to class (which can be a cropping management); the biological consistency is tested using a pairwise comparison procedure among subclasses through Wilcoxon rank-sum test (131). Finally, LDA (132) is performed to estimate the effect size of each differentially abundant feature. The data can be represented through bar graph or cladogram that also show the phylogeny of selected feature (Figure 4).

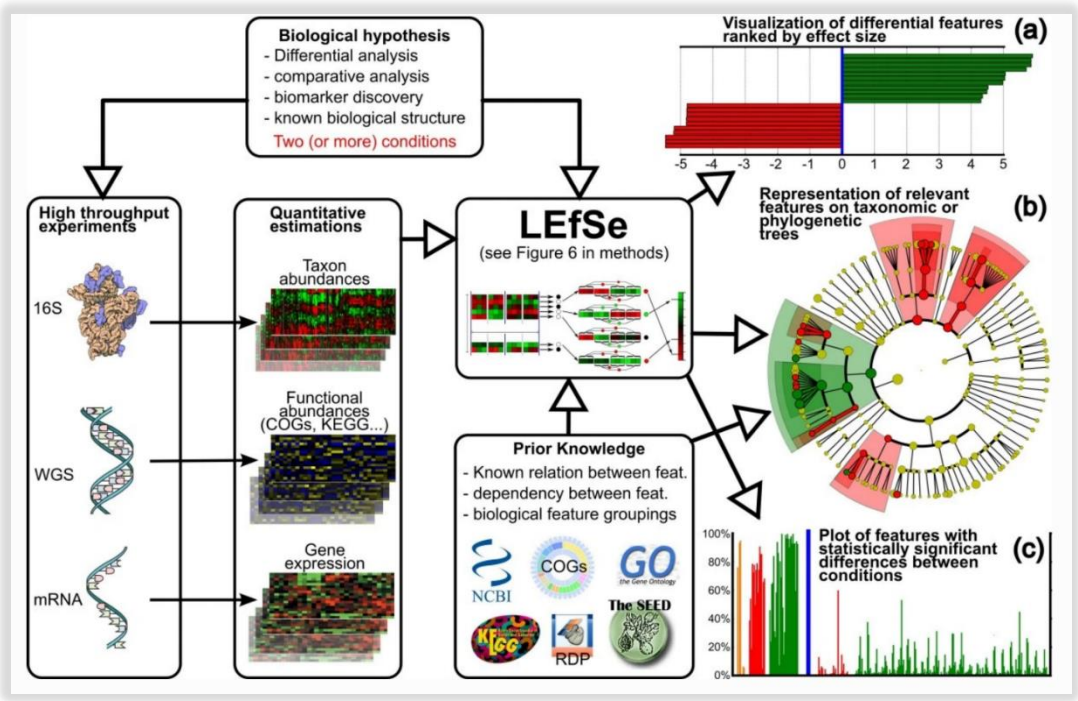


Figure 4. *Schematic LEfSe options (129) that is capable to identify and represent the taxon abundances, functional abundances and gene expression from amplicon, whole genome sequencing and mRNA.*

Other statistical techniques that study the differences using all the classifications or predicted genes is ***similarity percentage*** (SIMPER) ***analysis***, which determines the individual contribution of each specie based on the decomposition of Bray-Curtis dissimilarity index.

The above-mentioned techniques provide only information about soil microbial diversity. **Network analysis** can deep on ecological data, providing information about microbial community structure and their relationships. This approach explores large databases from high-throughput DNA sequencing technologies to discern relevant microorganisms in the community composition and functioning, as well as ecosystem transitions. Network analysis perspective is based on the graph theory of Operations Research, which is aimed to represent complex ecosystem processes and capture the interactions among environmental microorganisms such as mutualism, symbiosis, or competition. A molecular ecological network is a directed graph formed by nodes that represent each species connected by edges symbolising pairwise interactions (105). The extraction of the co-occurrence patterns by using network analysis can be performed through ***MENA pipeline***. Briefly, network construction is based on log-transformed relative abundance (Figure 5), and a similarity threshold (S_t) from the ***Pearson correlation matrix*** identified by a Random Matrix Theory (RMT) based approach for the feature selection. Topological network properties can be derived from the resulting stochastic network architectures to

characterise each topology and compare them, such as **connectivity** (number of edges between a node and other ones), **stress centrality** (number of geodesic paths passing the node), **clustering coefficient** (how well a node relates to its neighbours), **eigenvector centrality** (the degree of a central node connected to other central nodes) and other indexes like **average connectivity, average geodesic distance, geodesic efficiency, centralization of degree, average clustering coefficient** or **connectedness** (105).

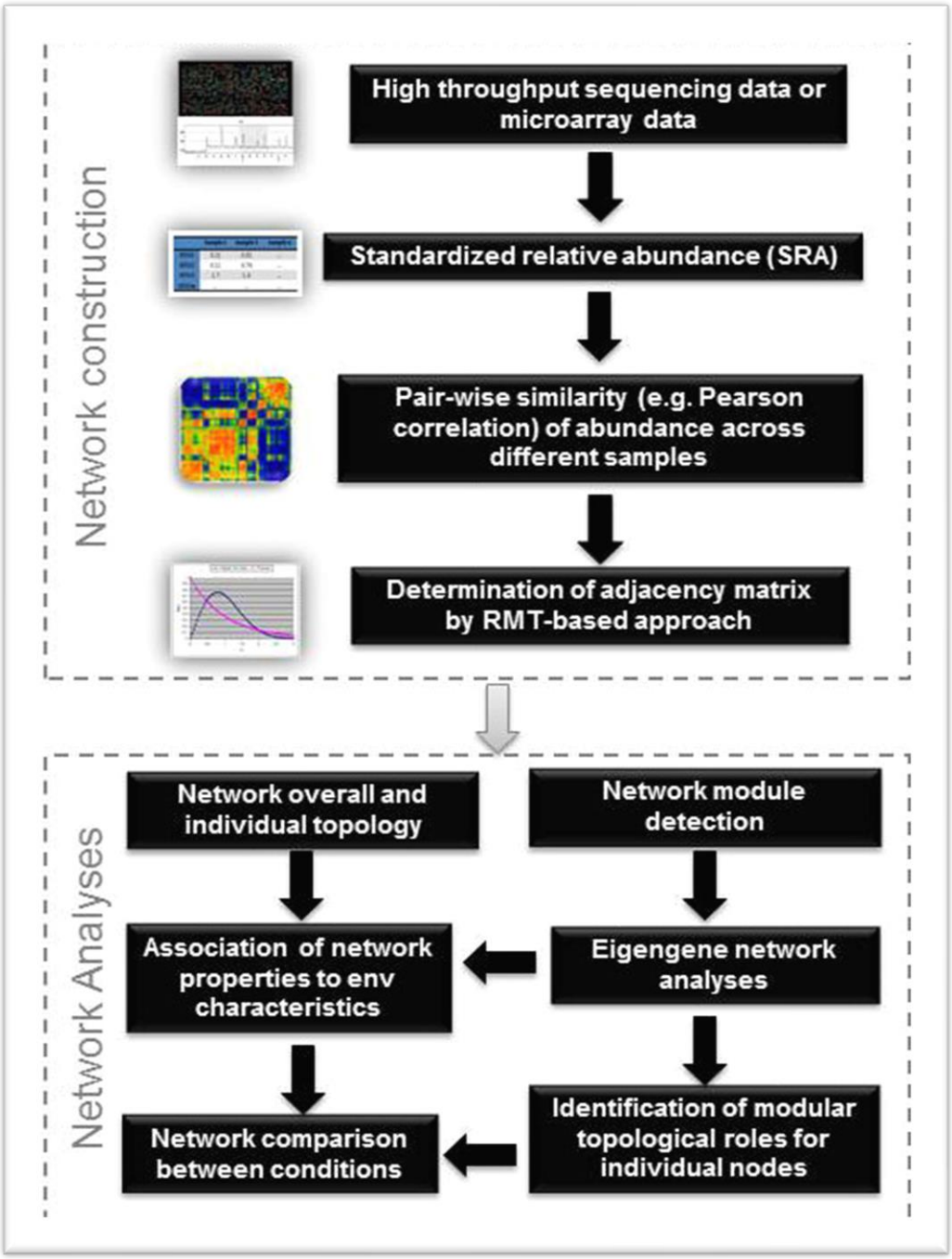


Figure 5. *Schematics steps for the network construction and network analyses in MENA (105).*

The modularity of the network is a very important concept in ecology because it could originate from specificity of the interactions (e.g., predation, pollination), heterogeneity of the habitat, ecological niche overlap, phylogenetic relatedness as well as stability and resilience (133). There are several methods to detect modules in a network such as **short random walk** (134), **leading eigenvector** (135), **simulated annealing** (136) and **greedy modularity optimization** (136), among the most used.

The network analysis involves an eigengene network analysis to reveal higher order organizations of the modules in the network structure (137,138). Each module is represented by a single representative abundance profile called module eigengene (137). Eigengene network is based on the correlations among module eigengenes, useful for identifying key populations of network topology (137); it can be represented using hierarchical clustering diagram and heatmap (105). The nodes play distinct topological roles in the network (137) which are classified from two parameters, **within-module** and **among-module connectivities, respectively,** into four categories: **peripherals** (specialists), **network hubs** (super-generalists), **connectors**, and **module hubs** (both close to generalists) (133).

6.3. Statistical methods for analysing jointly data from laboratory-prepared soil samples and from high-throughput technology

Nowadays, it is not possible to explain or understand the overall interaction in soils, although statistical tools make good approximations, emulating and recreating some interactions (139), or even trying to recreate soil processes using novel models at microscale (140). Within this framework, RDA produces an ordination that summarizes the main patterns of variation in the response matrix which can be explained by explanatory variables. The matrix is commonly composed of bacterial community, or some specific taxon and the explanatory variables correspond to the soil physical or chemical properties (129,141).

Programs have been developed to detect the influence of soil properties on bacterial community through the best subset of environmental variables with maximum correlation with community dissimilarities (***bioenv correlation analysis***) (104) or fitting environmental vector or factor onto an ordination (***envfit***) (104). These two approaches are widely used; they are commonly represented through NMDS.

There are other computational tools that allow to specify even more such as ***MENA,*** which provides the relationships between microbial network topology and environmental characteristics, basically deriving the OTU significance (GS) from the square of Pearson correlation coefficient of OTU abundance profile with environmental traits (105). ***Mantel and partial Mantel test*** calculate the correlations between the connectivity and multiple GS of environmental traits identifying the interrelationships between network topology

and environmental changes. The module's response to environmental changes might well be evaluated through the correlations between module-based eigengenes and environmental factors (105).

6.4. Computational resources for statistical analysis

Currently, there are many algorithms implemented in different software resources that allow us to analyse ecological data. One of the most common user-friendly programs to perform statistical data analyses is the Statistic Package for the Social Sciences (***SPSS***) (142) which, although was developed for social studies, has been very well welcome in agronomy. There are also user-friendly specific programs for data and data analysis such as ***primer6*** (143) or more exclusive software for omics data analysis such as ***PUMAA*** (144), ***EzMAP*** (145), ***iMAP*** (146) or ***Krona*** (147) which are built into open-source pipelines such as ***Mothur*** or ***QIIME***, or other programs such as ***CALYPSO*** (148), ***Cytoscape*** or ***Mena*** that are open access pipelines, user-friendly and have support making it very useful tools for data analysis. It should be pointed out that although these computational resources have been widely used in part of the research currently published, they present as main limitation that they are not flexible. To overcome this drawback, there are other computational software tools such as R/Bioconductor that permit to be as flexible as possible and offer large number of available free packages and options depending on the field of research. There are specialized packages for metagenomics study such a ***vegan*** (104), ***vegetarian*** (149), ***metagenomeFeatures*** (150), ***ampvis2*** (151), ***ecospat*** (152), ***biodiversityR*** (153), ***iNEXT*** (154), ***phyloseq*** (155), ***microbiomeSeq*** (156), ***microbiome*** (157), ***tidyMicro*** (158), ***hillR*** (159), ***microeco*** (160), etc.

In this thesis, different approaches have been performed and the tool selection was made based on the type of data. As a brief summary of the computational resources and collection of R functions used in the present dissertation, Table 2 shows the ones used in soil property data; Table 3 those applied to the HTS data; Table 4 includes those that are used to analyse jointly both types of data.

Table 2. *Software resources used for analysing soil properties data.*

RESOURCE/ FUNCTION	DESCRIPTION	SOURCE
aov	Compute the ANOVA test	R: stats
biplot	Perform a biplot of multivariate data	R: stat
boot	Generate bootstrap replicates of a statistic applied data	R: boot
DunnettTest	Perform a Dunnett's tests for comparing several treatments with a control	R:DescTools
dunnTest	Compute the multiple comparisons for a significant Kruskal-Wallis test with Bonferroni-Holm p-adjust method	R: FSA
Get_summary_stats	Compute summary statistics	R: rstatix
ggplot	Compute different graphs such a violin plots, boxplots, or bar plot	R: ggplot2
kruskal.test	Compute a Kruskal-Wallis rank sum test	R: stats
LeveneTest	Compute the Levene's test for homogeneity of variance	R: car
oneway.test	Compute the Welch's test	R: stats
Pairwise.t.test	Compute the pairwise comparisons between group levels with Bonferroni-Holm p-adjust method	R: stats
pca	Compute a PCA	R: FactoMineR
set.seed	Set the seed of random number generator to produce random samples	R: base
Shapiro.test	Compute the Shapiro-Wilk test for normality	R: stats
TukeyHDS	Compute the Tukey's Honest significant difference test	R: stats

Table 3. *Software resources used for analysing HTS data.*

RESOURCE/ FUNCTION	DESCRIPTION	SOURCE
ACACIA	Compute a denoising, filtering and clustering sequences	ACACIA
adonis	Compute an analysis of variance using distance matrices	R: vegan
anosim	Compute an analysis of similarities	R: vegan
aov	Compute the ANOVA test	R: stats
betadisper	Computes a Marti Anderson's procedure for the analysis of multivariate homogeneity of group dispersions	R: vegan
biom summarize-table	Compile a summary of the information of an OTU table	QIIME
boot	Generate bootstrap replicates of a statistic applied data	R: boot
ChaoRichness	Compute the estimation of species richness throug chao	R: iNEXT
Cytoscape	Open-source software for complex network visualization	Cytoscape
dada2	Perform the raw data denoising	QIIME2
diversity	Compute the Shannon, Simpson and Fisher diversity indices and species richness	R: vegan
estimateR	Find the number of unobserved species	R: vegan
fastqC	Quality control tool of sequences	fastqC
filter_otus_from_otu_table	Compute a filter to remove low confident OTUs	QIIME
Games-Howell	Compute a post hoc Games-Howell test	R
Get_summary_stats	Compute summary statistics	R: rstatix
ggplot	Compute different graphs such a violin plots, boxplots or bar plot	R: ggplot2
kruskal.test	Compute a Kruskal-Wallis rank sum test	R: stats
LEfSe	Compute the LEfSe analysis	Galaxy
LeveneTest	Compute the Levene's test for homogeneity of variance	R: car
MENA	Compute a co-occurrence network analysis	MENA
metaMDS	Compute a nonmetric multidimensional scaling	R: vegan
myplotbetadisper	Perform a PCoA graph	R: vegan
oneway.test	Compute the Welch's test	R: stats
Pairwise.t.test	Compute the pairwise comparisons between group levels with Bonferroni-Holm p-adjust method	R: stats
pick_open_reference_otus	Compute a pipeline for OTU picking, phylogenetic tree construction, taxonomic classification…	QIIME
qiime demux summarize	Summarize a sample data	QIIME2
Qiime feature-classifier	Perform the classification of the taxa	QIIME2
qiime feature-table	Remove features from OTU table	QIIME2
qiime taxa filter	Remove taxa from taxonomic table	QIIME2
qiime tools import	Import the sequences into QIIME2	QIIME2
Qiime_vsearch	Perform the OTU clustering	QIIME2

cluster		
set.seed	Set the seed of random number generator to produce random samples	R: base
Shapiro.tes	Compute the Shapiro-Wilk test for normality	R: stats
simpe	Discriminate species between groups using Bray-Curtis dissimilarities	R: vegan
single_rarefaction	Compute a subsampled OTU table	QIIME
split_libraries	Compute a quality filtering, trim primers, and adaptors	QIIME
summarize_taxa	Provide summary information of the representation of taxonomic groups within each sample	QIIME
TukeyHDS	Compute the Tukey's Honest significant difference test	R: stats
vsearch -uchime_ref	Compute a PCR chimera detection using vseach	QIIME

Table 4. *Software resources used for analysing jointly HTS data and soil properties.*

RESOURCE/ FUNCTION	DESCRIPTION	SOURCE
bioenv	Compute the best subset of environmental variables with maximum rank correlation with community dissimilarities	R: vegan
cor.test	Compute Spearman/Pearson correlation test	R: stats
envfit	Fit an environmental vector of factor into an ordination	R: vegan
Get_summary_stats	Compute summary statistics	R: rstatix
ggplot	Compute different graphs such a violin plots, boxplots or bar plot	R: ggplot2
MENA	Compute a co-occurrence network analysis	MENA
plot	Plot a graph	R: graphics
rda	Compute a RDA	R: vegan
sample_n	Select a random number of rows (samples)	R: dplyr
set.seed	Set the seed of random number generator to produce random samples	R: base

Publications

Changes in Bacterial and Fungal Soil Communities in Long-Term Organic Cropping Systems

Abstract

Organic farming that includes the use of organic fertilizers such as compost or manure and techniques such as crop rotation are of growing interest. The important role that microorganisms play in the soil and understanding how they respond to organic farming and their relationship with soil properties, can be resulted useful for farmers to contribute to improve soil health and crop quality.

The aim of this paper is to investigate the potential differences between organic systems (organic cultivation with manure compost and compost tea (Org_C) and organic cultivation with manure (Org_M)) and a conventional system (Conv). For this purpose, in the three studied agricultural systems, the soil bacterial and fungal communities are deeply studied through high-throughput sequencing analysis for bacterial (16S), fungal (ITS) community, and also fungal plant pathogens measured by qPCR; their relationship with soil properties and crop production are evaluated through different statistical tests.

To detect those soil properties on which the cropping system has a significant effect, Analysis of Variance (ANOVA) or Kruskal-Wallis tests are performed followed by pairwise comparisons (Tukey's or Dunn's test). To know the biodiversity of microbial community in the different cropping systems, both α- and β- diversities are calculated and their differences are tested through ANOVA, when normality and homoscedascity are fulfilled, and Permutational Multivariate Analysis of Variance (PERMANOVA), when the homogeneity of variance is fulfilled, and otherwise using Kruskal-Wallis test and Analysis of Similarities (ANOSIM). The most abundant phyla and genus stablished in each cropping system and their differences are also tested through ANOVA or Kruskal-Wallis test depending on whether the data meet the assumptions or not. A linear discriminant analysis Effect Size (LEfSe) is employed using all the taxonomic classifications, with the aim to detect microbial biomarkers under each of the studied cropping system. The Similarity Percentages (SIMPER) statistical method is performed to know the contributions of each taxon (at phylum level) to the differences among the cultivation system. The relationships between microorganisms and soil properties are studied through Spearman correlation analysis; the ordination projection of soil properties are represented by Nonmetric Multidimensional Scaling (NMDS).

The ANOVA shows that crop yield is similar in the two organic cultivation systems, with no differences with the conventional one. Bacterial α-diversity shows no significant differences among the cropping systems, although fungal does. Both bacterial and fungal community structures are mapped by Principal Coordinates Analysis (PCoA); the significant effect of the cropping systems on each community is confirmed by the respective PERMANOVA. A NMDS of the bacterial and fungal community structures and the significant soil parameters report that total organic carbon, total nitrogen, total ammonium, Mg and B show significant effect on bacterial community but not on fungal community. A LEfSe analysis discloses different bacteria and fungi as key microorganisms for each of the three different cropping systems. The long-term application of pesticides in the conventional system favoured the higher abundance of microorganisms associated with the pesticides, such as *Nesterenkonia, Galbibacter, Gramella, Limnobacter, Pseudoalteromonas, Pantoe,* and *Sporobolomyces.* For the two organic systems, other types of microorganisms such as *Terrimicrobium*, *Galbibacter*, *Turicibacter*, *Aciditerrimonas*, *Nibribacter*, *Haliangium, Candida*, *Wallemia* or *Funneliformis* are characterized to be associated to organic amendments; they could be involved in different soil functions, such as the reduction of soil borne pathogens (*Haliangium* or *Wallemiales*).

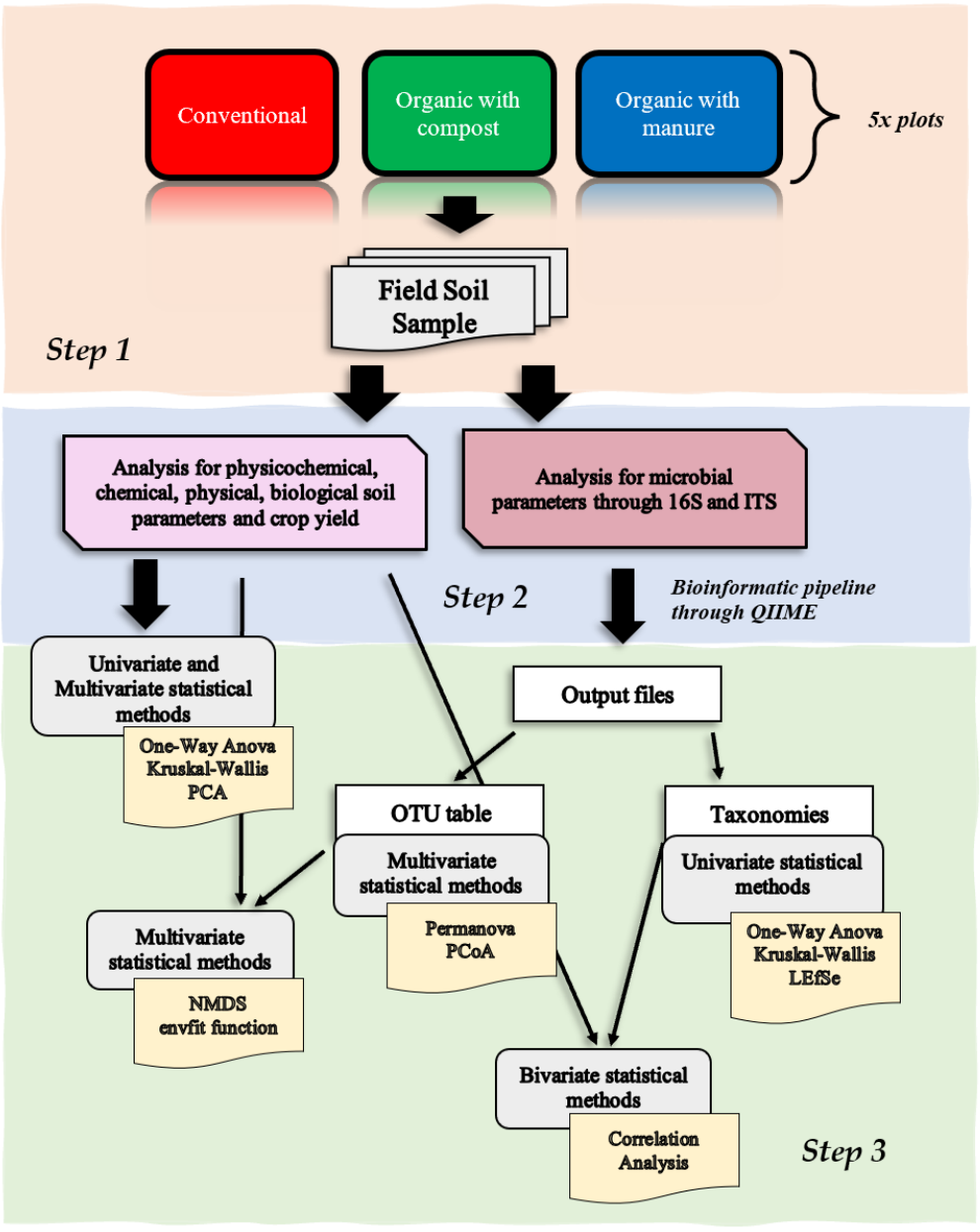
Relevant contributions reached with this paper are that cropping system influences significantly on soil properties and bacterial and fungal communities, and that the type of cropping system promotes the growth or loss of some specific taxa; it is the case of conventional cropping system where there is a bias towards species more tolerant to the high amount of pesticides and herbicides, an increase that could be due to the loss of more sensitive species, being able to promote the disappearance of beneficial species that have been observed on soil where organic amendments have

been incorporated; that could contribute to reduce the soil-borne disease or the increase of soil nutrients.

This study also shows that organic farming, instead of conventional farming system, is a suitable sustainable agriculture system, and the use of compost as a long-term organic amendment (Org_C) do not show differences on crop yield with the other systems, but it shows a stable system, with a high total organic carbon and nutrients available by plants, as well as a change in the bacterial and fungal communities

Further studies should examine the possible mechanisms, behind microbial community changes related to specific biogeochemical cycles, as well as the functional approach and inter-connection between microbial communities, associated with organic matter decomposition with different putative functions.

Workflow


Conventional
Organic with compost
Organic with manure
5x plots
Field Soil Sample
Step 1
Analysis for physicochemical, chemical, physical, biological soil parameters and crop yield
Analysis for microbial parameters through 16S and ITS
Step 2
Bioinformatic pipeline through QIIME
Univariate and Multivariate statistical methods
One-Way Anova
Kruskal-Wallis
PCA
Output files
OTU table
Taxonomies
Multivariate statistical methods
Permanova
PCoA
Univariate statistical methods
One-Way Anova
Kruskal-Wallis
LEfSe
Multivariate statistical methods
NMDS
envfit function
Bivariate statistical methods
Correlation Analysis
Step 3

agriculture

MDPI

Article

Changes in Bacterial and Fungal Soil Communities in Long-Term Organic Cropping Systems

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Abstract: Long-term organic farming aims to reduce synthetic fertilizer and pesticide use in order to sustainably produce and improve soil quality. To do this, there is a need for more information about the soil microbial community, which plays a key role in a sustainable agriculture. In this paper, we assessed the long-term effects of two organic and one conventional cropping systems on the soil microbial community structure using high-throughput sequencing analysis, as well as the link between these communities and the changes in the soil properties and crop yield. The results showed that the crop yield was similar among the three cropping systems. The microbial community changed according to cropping system. Organic cultivation with manure compost and compost tea (Org_C) showed a change in the bacterial community associated with an improved soil carbon and nutrient content. A linear discriminant analysis effect size showed different bacteria and fungi as key microorganisms for each of the three different cropping systems, for conventional systems (Conv), different microorganisms such as *Nesterenkonia, Galbibacter, Gramella, Limnobacter, Pseudoalteromonas, Pantoe*, and *Sporobolomyces* were associated with pesticides, while for Org_C and organic cultivation with manure (Org_M), other types of microorganisms were associated with organic amendments with different functions, which, in some cases, reduce soil borne pathogens. However, further investigations such as functional approaches or network analyses are need to better understand the mechanisms behind this behavior.

Keywords: compost; high-throughput sequencing; sheep manure; soil properties; crop yield; organic farming; microbial community structure

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1. Introduction

Currently, one third of agricultural soils worldwide are moderately or highly degraded, thus affecting production [1]. Chemical fertilizers, pesticides, and herbicides are 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, as well as a decrease in soil biodiversity [3]. In this context, organic farming is of growing interest. Organic farming includes the use of organic fertilizers, such as compost, manure, or green manure, and places an emphasis on techniques like rotation with companion plants or intercropping, and pest and disease control by natural methods, avoiding synthetic

chemical compounds, preserving the environment, and providing human beings with high-nutrition crops that are free of chemicals [4]. 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 [5], with more than 2.2 million hectares, accounting for 16.7% of the total farmed land in the country.

Some studies have reported 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, recent studies have highlighted that long-term organic management can contribute crop yields similar to those found in conventional farming, once the system has been stabilized, after initial years with a reduction in crop yields [7,8]. For this reason, more information about the relative variability of organic systems compared with conventional ones is necessary in order to reinforce the use of organic farming.

Soil microorganisms play an important role in ecosystem processes, such as carbon cycling, decomposition, nutrient cycling, and soil aggregate formation [9], and determining this relationship is very complex [10]. Understanding how these microorganisms respond to organic matter, inorganic fertilizers, and soil management can help farmers to improve soil health for crop production [10–12]. Approaches like high-throughput analysis of bacterial and fungal communities can show taxonomic shifts, shaping the patterns of the ecological interactions that regulate the structure, function, and resilience of soil microbial communities under organic farming compared with conventional farming.

However, there are no conclusive results regarding this. Some studies have reported that after long-term organic farming, the microbial diversity, soil sustainability, and beneficial microorganisms involved in plant health were higher than in conventional farming [13–15]. However, Bell et al. [16] and Krishnaraj and Sabale [17] found that organic cropping systems showed no significant differences, or showed an even lower microbial diversity, compared with conventional cropping. So, we found a gap of knowledge that needs more results from long-term field experiments, such as the one presented here, to know how soil microbial communities change across different organic farming types.

The aim of this paper was to investigate the potential differences between two organic systems (organic cultivation with manure compost and compost tea (Org_C) and organic cultivation with manure (Org_M) with a conventional system (Conv). For this purpose, in the three studied agricultural systems, the soil bacterial and fungal communities were studied through high-throughput sequencing analysis, focusing and studying their rela- tionship using physico-chemical soil properties and crop production. We hypothesized that (a) organic systems would not have significant differences on crop production compared with the conventional system, as once an organic system is stabilized, soil functionality is improved, contributing to a high nutrient availability and soil health; (b) both organic systems (Org_C and Org_M) would promote changes in the microbial structure and abundance compared with the 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 south-eastern Spain. The soil was a Haplic Calcisol (Loamic, Hypercalcic) IUSS [18]. The area has a mean annual temperature of 17.5 °C, 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, where the following five random plots (~1 ha) were set up for each cropping system: (1) a conventional system using a yearly addition of sheep manure as an organic amendment, inorganic fertilizer for fertigation, and

pesticides (Conv); (2) an organic system using a yearly addition of compost, amino acids to provide N, compost tea to provide organic compounds and nutrients for fertigation, no pesticides, and the use of cover crops of oat (*Avena sativa)* and vetch (*Vicia sativa)* between cropping seasons (Org_C); and (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. One composite sample derived from ten subsamples (0–10 cm depth) was collected using an auger from each plot in February 2018 after the harvest of a leaf cabbage crop (*Brassica olearacea var. sabellica*).

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; 10 L 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 24 h.

The characteristics of the sheep manure, compost, and tea compost are shown in Table S1. Sheep manure and its compost were chosen because of their proximity to the experimental plot, thereby reducing the environmental and economic impact of their transportation to the experimental site. 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 for 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).

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 a molecular analysis. The samples were taken to the lab immediately. The soil for the molecular analysis was sieved at <2 mm and stored at −20 °C. The soil was air-dried for one week for the physicochemical analyses and sieved at <2 mm. The soil cores were taken using steel cylinders to determine the soil bulk density [19].

2.2. Soil Properties and Crop Yield

The actual field soil moisture content (FMa) was gravimetrically determined according to De Angelis [20]. The cation exchange capacity (CEC) and exchangeable Ca^{2+}, Mg^{2+}, K^{+}, and Na^{+} were determined using BaCl2 as the exchangeable cation, following ISO (international standard method) 13536 [21]. The soil water content at wilting point (SWW) and soil water content at field capacity (SWFC) were calculated using the retention curve method [22], in which moist samples were dried by raising the air pressure in an extractor with a porous ceramic plate [23]. 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 using an elemental CHNS-O analyzer (EA-1108, Carlo Erba, Barcelona, Spain), and the $CaCO_3$ content was calculated from the IC. The particulate organic carbon (POC), defined as a fresh or decomposing organic material, was measured according to Cambardella and Elliot [24]; 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 [25]. Soil NH_4^{+} was extracted with 2M KCl in a 1:10 soil/extractant ratio, and was calorimetrically measured [26,27]. Soil NO_3^{-} was extracted with deionized water in a 1:10 soil:extractant ratio and was measured by ion chromatography (Metrohm 861). The available P (P) was measured using the Olsen method [28]. The available Fe, (Fe) Mn (Mn), Cu (Cu), and Zn (Zn) were extracted by chelation using DTPA (1:2 *w/v*) [29,30]. The available B (B) was extracted with deionized water (1:5 *w/v*) at 50 °C [31]. The available nutrients were measured using ICP-MS (7500CE, Agilent, Santa Clara, CA, USA). The total pesticides were determined with the QuEChERS method [32], according to which, 5 g of a homogenized sample was extracted with 10 mL acetonitrile containing 1% acetic acid. The pesticides were analyzed through liquid chromatography triple quadrupole mass spectrometry (LC-MS/MS; TQS MS linked to a Waters Acquity UPLC system, Waters Corporation, Milford, MA, USA) and gas chromatography triple quadrupole mass spectrometry (GC-MS/MS; Agilent 7890B GC coupled to an Agilent 7010B MS system, Santa Clara, CA, USA).

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, Hilden, Germany), following the manufacture's protocol, with the modifications described by Taskin et al. [33]. The quantity and quality of DNA extracts were quantified using a Qubit 3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, Waltham, MA, 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, London, UK) System. Bacterial 16S regions were amplified using an Ion 16S™ Metagenomics Kit (Thermo Fisher Scientific, Waltham, MA, USA) with two different degenerate primer sets to amplify regions V2–4–8 and V3–6, V7–9. The 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, Waltham, MA, USA).

All of the purification processes between incubation and the amplification reactions of library preparation were processed using DynaMag™-2 magnetic racks (Thermo Fisher Scientific, Waltham, MA, USA) and an AMPure XP Purification Kit (Beckman Coulter, Brea, CA, USA). Library preparation and barcoding were followed by the determination of the size and concentration of the final libraries using an Agilent 2100 Bioanalyzer system and the Agilent High Sensitivity DNA kit (Agilent, Santa Clara, CA). Sequencing templates were prepared using an Ion One Touch 2 System and an Ion PGM™ Hi-Q™ View OT2 Kit (Thermo Fisher Scientific, Waltham, MA, USA). The sequencing reaction was performed using Ion Torrent PGM (Thermo Fisher Scientific, Waltham, MA, USA) with an Ion PGMTM Hi-QTM View Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA).

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 (Illumina, San Diego, CA, USA). The reverse primers were barcoded using the 12-base Golay barcodes [35]. DNA extraction had a minimum concentration of 10 ng/µL with a least 200 ng provided. The PCR amplifications were conducted in a final volume of 30 µL containing 3 µL of buffer 10×, 0.7 µL of each primer (10 mM), 0.9 µL of 50 mM $MgSO_4$, 0.6 µL of 10 mM dNTP, 2 µL of template DNA (10 ng/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 °C, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 50 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR products were cleaned up from primers using Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, IN, USA), following the manufacturer's instructions. The PCR products were checked on a Bioanalyzer DNA 1000 kit (Agilent, Santa Clara, CA, USA) to verify the size. Amplicons were quantified with Qubit using the dsDNA HS Assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Amplicon libraries were sequenced on the Illumina MiSeq machine (Illumina, San Diego, CA, USA), together with a 10% PhiX (Illumina, San Diego, CA, USA), control library to generate 300 bp paired end reads.

2.4. Sequencing Data Processing

For bacterial raw sequences, the barcodes and primers were trimmed according to the BaseCaller software (ThermoFisher, Waltham, MA, USA). The sequences were denoised with ACACIA v 1.53 [36], and low quality sequences were discarded using the Quantitative Insights into Microbial Ecology (QIIME) pipeline v 1.9.1 [37] from the Microbiome Helper Virtual Box v 2.3 [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 128 after filtering chimeras using VSEARCH v 2.4.3 [39] with the ribosomal database project (RDP_trainset16_022016.fa) [40]. Low confidence OTUs were 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 using the Trimmomatic v 0.38 program [41], setting the quality cutoff to 20 in 24 bp sliding win- dows. Trimmed reads were assembled using the paired-end read merger (PEAR) pro- gram v 0.9.10 [42]. Chimeras were removed using VSEARCH v 2.4.3 from the QIIME pipeline v 1.9.1 using the UCHIME reference dataset (uchime_sh_refs_dynamic_origin- al_985_03.07.2014.fasta). OTUs were assigned based on 97% similarity using the open refer- ence OTU picking protocol implemented in the QIIME toolkit v 1.9.1. Taxonomy was as- signed using the UNITE database (sh_taxonomy_qiime_ver7_dynamic_20.11.2016.txt) [43]. Low abundance OTUs (OTUs with less than three 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, Foster City, CA, USA). The reaction mixtures (15 µL) contained a final concentration of 1 × TaqMan Universal Master Mix II no UNG (Applied Biosystems, Foster City, CA, USA), 0.3 µm of each primer, 0.1 µmof 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 °C for 10 min, followed by 40 cycles at 95 °C for 10 s, and at 60 °C for 40 s, as well as afinal step at 50 °C 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, Foster City, CA, USA). Fungal pathogen detection and quantification

were 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 a mean comparison between the 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 v 0.8.3 [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 v 1.42 [46], a PCA biplot was generated using the factoextra package v 1.0.5 [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 v 1.1.453 packages of iNEXT v 2.0.19 [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.

A similarity percentages (SIMPER) analysis was conducted using the "simper" function of the vegan package v 2.5.6 to identify the parameters that most contributed to the pairwise differences between the cropping systems at a phylum level. PERMANOVA was conducted to test the differences among the cropping systems if the homogeneity of variance assumption was met, and an analysis of similarities (ANOSIM) was carried out if not.

A linear discriminant analysis (LDA) effect size (LEfSe; Galaxy community hub https://huttenhower.sph.harvard.edu/galaxy/, accessed on 20 November 2020) under the default parameters was implemented to identify differentially abundant groups among the three cropping systems [50,51].

In order to visualize and test whether the microbial community structures (OTUs) of the three cropping systems were distinct, a 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 the soil properties and soil microbial community were assessed using 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

The 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 showedthe 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, and 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 the 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 the cropping systems (Table 2).

The obtained biplot reflected the differences among the three cropping systems (Figure 1). Org_C was associated with TOC, TN, B, Mg, and NH_4^+, contrary to Org_M, which was negatively correlated with POC (Figure 1). The Conv system was positively correlated with the total pesticide content.

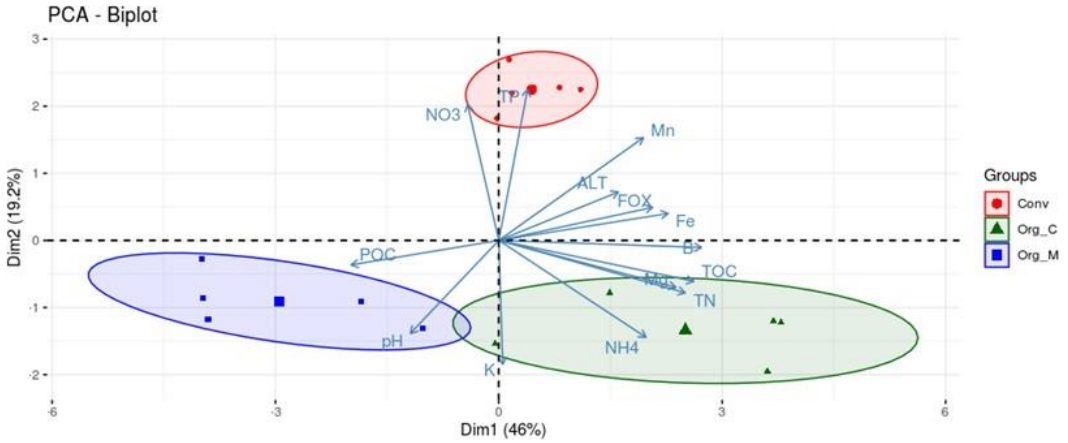


Figure 1. Principal component analysis (PCA) of the soil properties. The two first principal components are shown on the *x*- and *y*-axes, respectively. PCA scores represent soil samples, with colors indicating the corresponding cultivation systems. Ellipses represent 70% confidence intervals around the barycenters for the 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 the cabbage yield among the three cropping systems (Figure 2).

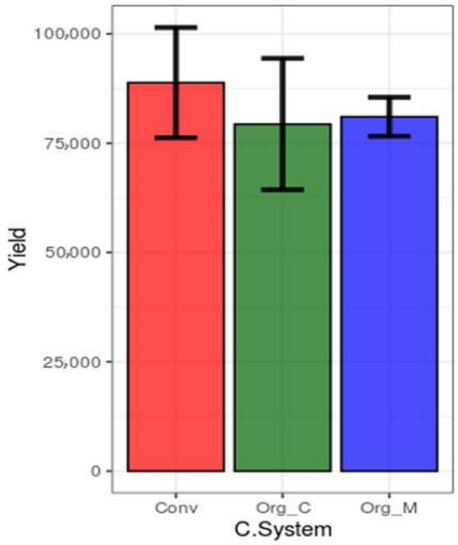


Figure 2. The crop yield (kg ha^{-1}) of the three cropping systems. Error bars represent mean ± standard deviation (SD; *n* = 5). No significant differences were found among the three cultivation systems (Kruskal–Wallis test, $p > 0.05$). Conv—conventional system; Org_M—organic cultivation system with 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 of the 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 the microbial communities (Figure 3 and Table S2). The Chao1 index showed no significant differences in the bacterial and fungal communities among the three cropping systems (Figure 3 and 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 and Table S2).

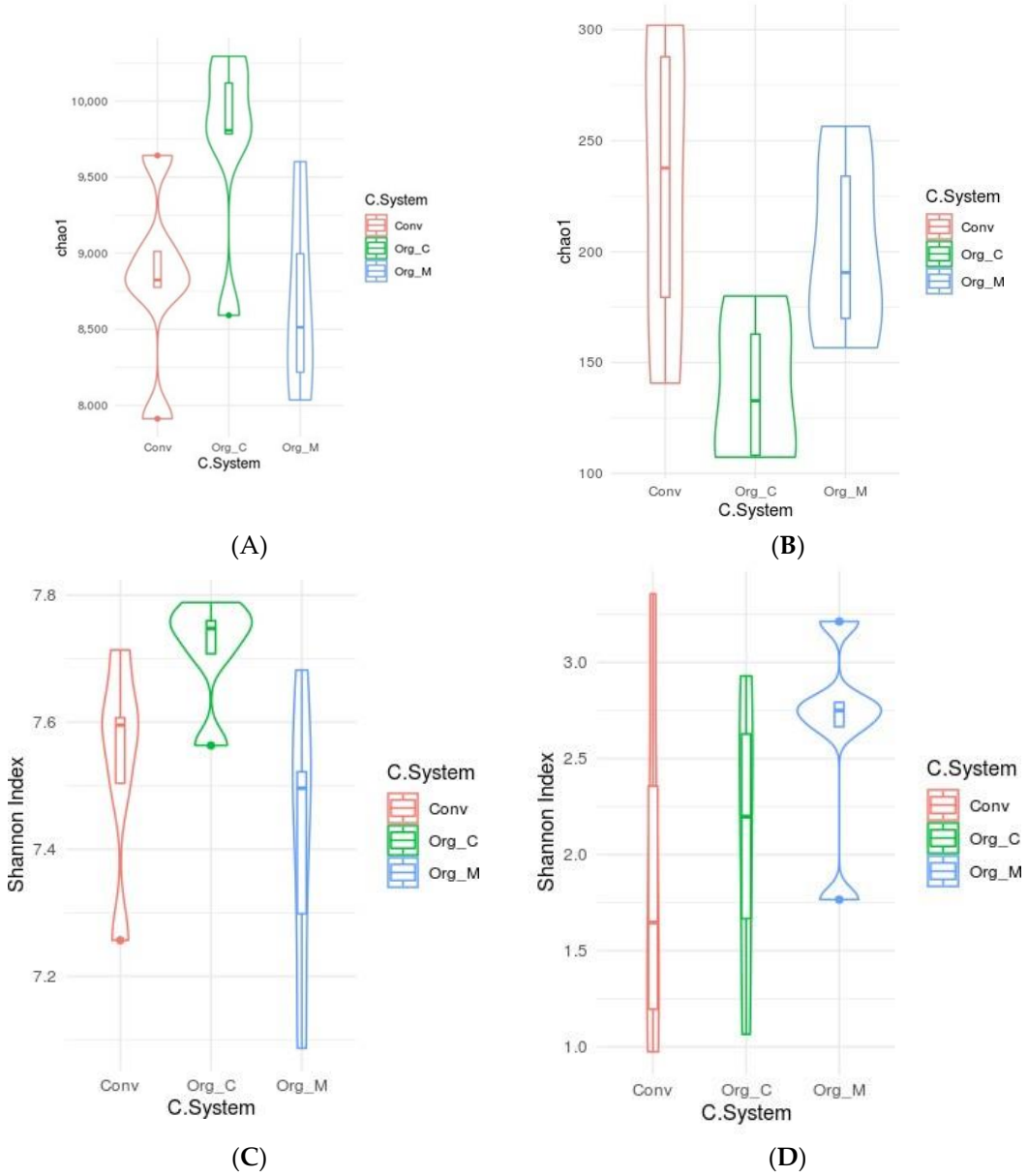
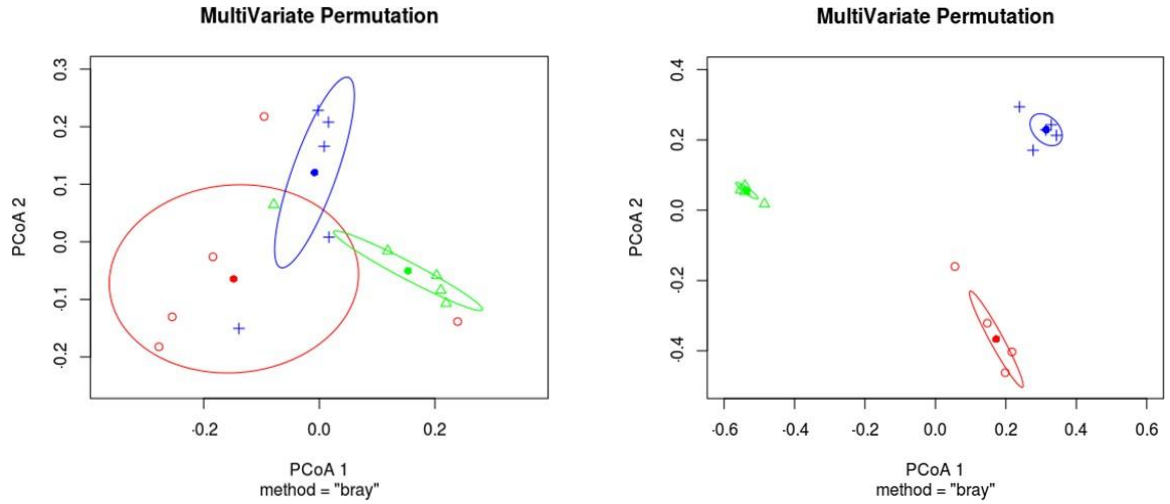


Figure 3. Violin plots displaying the diversity indexes in the three cultivation systems. The distributional features of the data are depicted by the kernel density trace overlaid on the descriptive statistics (median and whisker range from 25% to 75%) represented by a boxplot. Chao1 for (**A**) bacterial and (**B**) fungal communities, and Shannon index for (**C**) bacterial and fungal (**D**) communities. 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).

The PCoAs of the bacterial (Figure 4A) and fungal (Figure 4B) microbial communities showed significant differences among the different cropping systems, which were confirmed by PERMANOVA (F = 1.792, P = 0.006; F = 7.649, and P = 0.001 respectively).

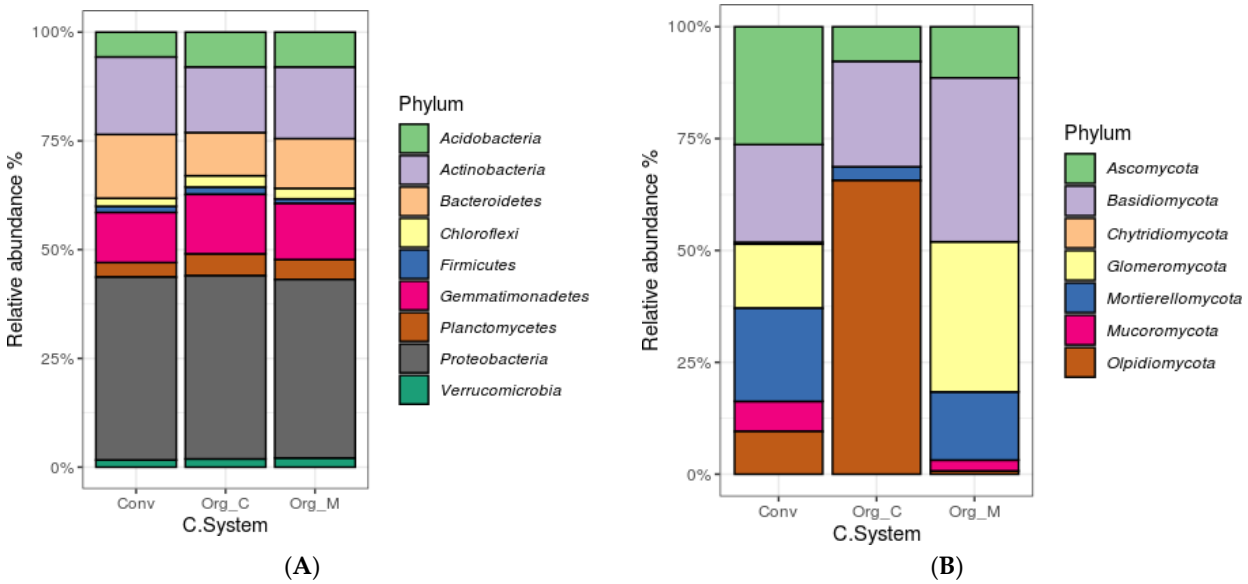


(**A**) (**B**)

Figure 4. Principal coordinate analysis (PCoA) of the (**A**) bacterial and (**B**) fungal community structures between the three cropping systems. Different colors and shapes represent the different points of the group—Conv is indicated by red circles, Org_C by green triangles, and Org_M by blue crosses. PCoA displays the 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).

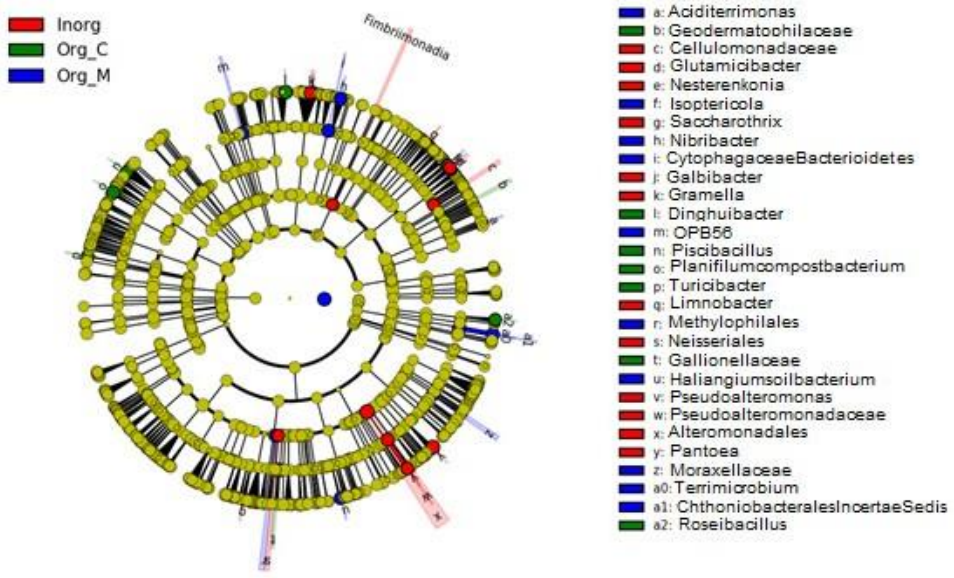
3.3. Effects of Different Cropping Systems on Bacterial and Fungal Community Composition

The main bacterial and fungal taxa found in the different cropping systems are shown in Figures 5–7. The most dominant phyla under the different cropping systems were Proteobacteria (42% in average), Actinobacteria (16%), Bacteriodetes (12%), and Acidobacteria (7%; Figure 5A and Table S3). No significant differences were found among the three cropping systems when using Bray–Curtis dissimilarity at a 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.

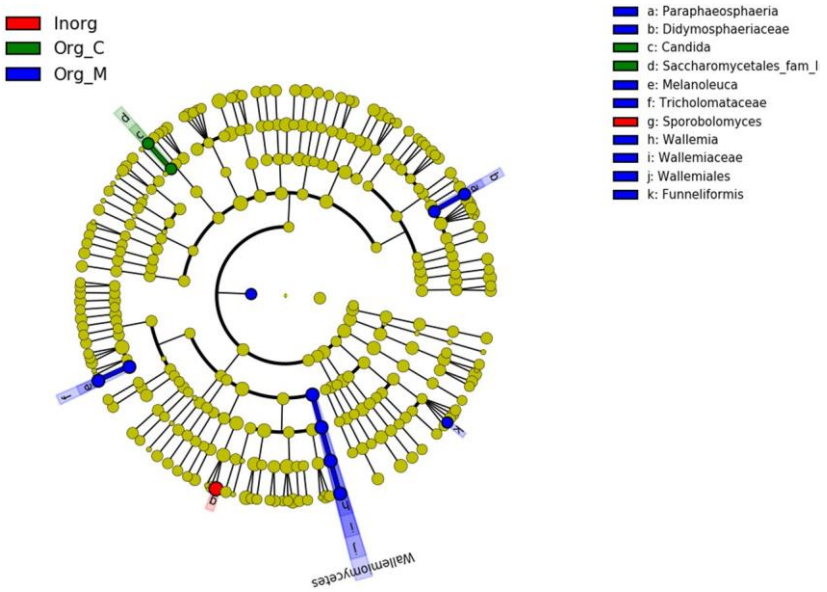


(**A**) (**B**)

Figure 5. Relative abundance (>1%) of (**A**) bacterial and (**B**) fungal phylum of the three cropping systems. Bar values are mean ± SD (n = 5). Conv—conventional system; Org_C—organic cultivation with manure compost and tea compost; Org_M—organic cultivation with manure.

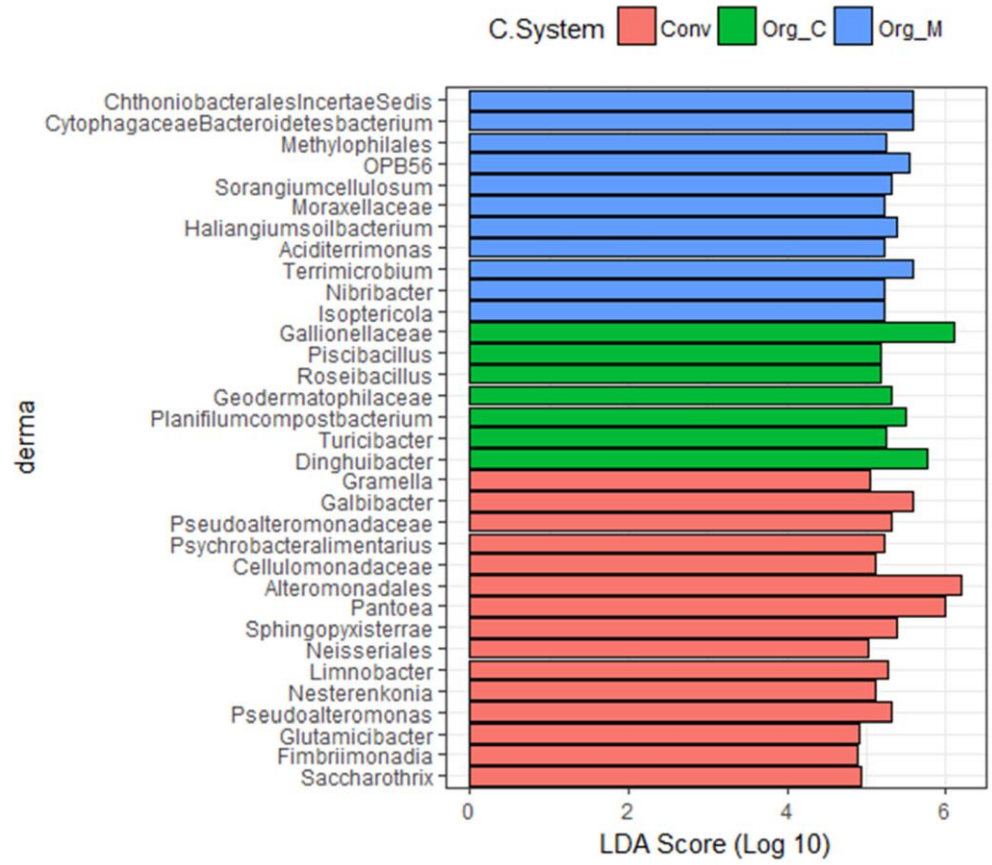


(**A**)

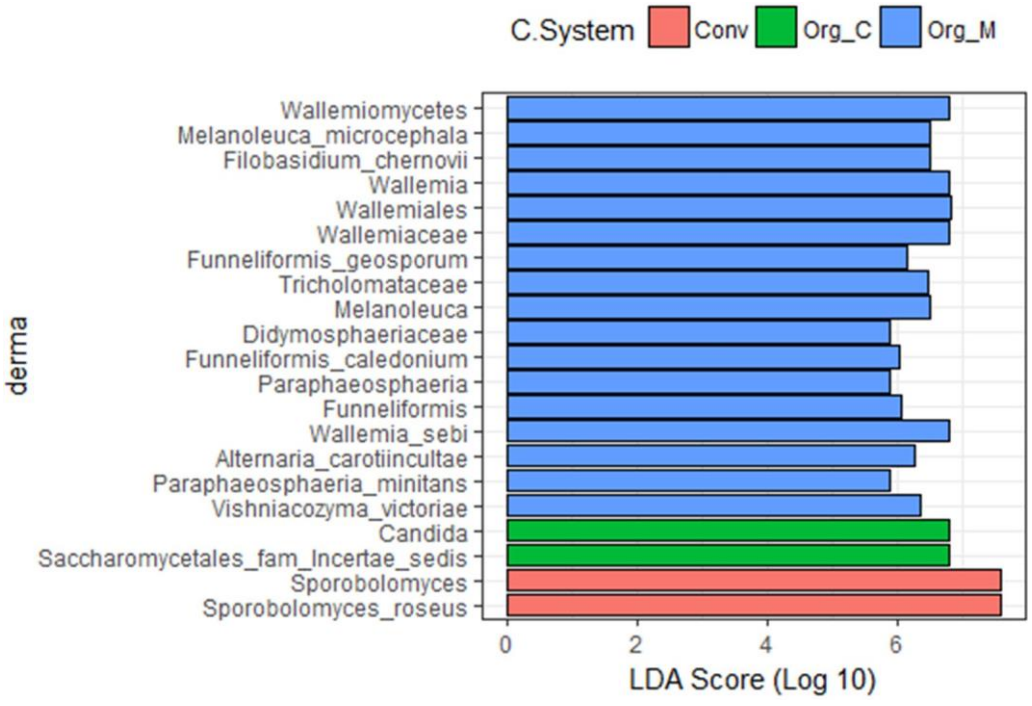


(**B**)

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 and (**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).

The most abundant fungal phylum was Basidiomycota (27% on average), followed by Olpidiomycota (25%), Ascomycota (15%), Glomeromycota (15%), and Mortierellomycota (13%; Figure 5B and 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 the 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 (Figures 6A and 7A). At the genus level, *Nesterenkonia* (Actinobacteria); *Galbibacter* and *Gramella* (Bacteroidetes); and *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 (Figures 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 (Figures 6B and 7B). The genus *Sporobolomyces* (Basidomycota) was the only genomic feature in the Conv system. For the organic systems, we found the genera *Wallemiales* (Ba- sidiomycota), *Funneliformis* (Glomerales), *Melanoleuca* (Basidiomycota), and *Alternaria* and *Paraphaeosphaera* (Ascomycota) in Org_M, and the genus *Candida* (Ascomycota) in Org_C.

3.4. The Relationship between the Microbial Community and Soil Properties

A nonmetric multidimensional scaling (NMDS) was assayed for establishing the relationship between bacterial and fungal communities and the significant soil parameters. For bacteria, TOC, TN, NH_4^+, Mg, and, B were the soil properties that showed a significant effect on the bacterial community composition (Table S5). However, for fungi, no significant correlation was found (Table S6).

A Spearman correlation analysis between the 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 NH_4^+ (0.60 *, 0.66 **, 0.59 *, 0.74 **, 0.77 **, 0.76 **, and 0.70 **, respectively); *Aciditerrimonas, Roseibacillus*, and *Nibribacter* showed a high correlation with TOC (0.82 ***, 0.74 **, and −0.65**, respectively) and *Aciditerrimonas* with total nitrogen (0.64 **); *Terrimicrobium* with NH_4 * (−0.58 *); and *Wallemia* with both pathogens *Alternaria* spp. (−0.77 **) and *Fusarium oxysporum* (−0.56 **) and with NH_4 * (−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. The 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 the higher organic carbon content in Org_C may have contributed to the

slightly lower pH in soil compared with Org_M, owing to the greater presence of organic acids [54,55]. 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. Meanwhile, 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 the pH [56]. On the other hand, the higher NO_3^- levels observed in the Conv system were probably due to intensive chemical fertilizer and pesticide use, and the addition of nitric acid in fertigation [57,58].

It is interesting to highlight that soil parameters such as TOC and some nutrients like TN, Mg, and B were associated with changes in the soil bacterial community, as observedby Yang et al. [59] and Zhang et al. [60]. It is well known that compost enhances carbon and nitrogen in soils, thus changing the microbial communities [61]. In addition, Vera et al. [62] observed that the boron content in the soil was a determinant property explaining the changes in the bacterial community in agricultural soil. According to Vera et al. [63], organic matter had a key influence on the potential microbial action with high boron doses, and our findings could support that influence, as 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 [64]. It was contrary to the lower values expected in conventional systems due to the adverse effects of agrochemicals [65–67]. Moreover, a significantly higher fungal diversity was observed in the Conv system, probably due to the increase in nutrient availability, as previously observed by Geisseler and Scow [68] and Leff et al. [69], and the breakage of fungal hyphae by tillage [70]. 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 [71].

Most importantly, our results showed that there are different effects from the various 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 [72]. The effect of the different cropping systems was not reflected in the dominant bacterial phyla, contrary to that observed by Lupatini et al. [71] or Moreno-Espíndola et al. [73]. Hartman et al. [72] 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 relative abundance of Ascomycota and Mortierellomycota markedly increased in response to the fertilizer treatments. Ascomycota and Mortierellomycota rapidly metabolize rhizodeposited organic matter in rhizosphere soil, so their abundances are stimulated by nutrient substance [74].

Hence, fertilizer management may result in suitable circumstances for phyla that obtain sufficient levels of C, N, and P from the top soil [75].

Basidiomycota and Glomeromycota had the greatest dissimilarity in Org_M compared with Conv. Previous studies showed that Basidiomycota was increased in soils with manure as it provides an appropriate environment for Basidiomycota [76]. However, chemical fertilizer can cause the loss of that environment and can hinder the development of this phyla [76]. A high abundance of Glomeromycota in Org_M can form arbuscular mycorrhiza with plants and absorb nutrients directly, particularly P uptake through the plant roots [77], promoting plant growth, and enhance plant resistance to various pathogens [78], 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 some pathogens species of Olpidyomicota (*Olpidium*) in subsequent crops could be determined to cause disease on some of them, along with certain environmental factors [79]. Furthermore, Carini et al. [80] 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 provide a more meaningful assessment of cellular viability and plant infection [79].

The presence of several differentially taxa among cropping systems provides information on soil microbiota responses to different agricultural management practices [81]. According to the LEfSe analysis, the long-term application of pesticides in the 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 [82]. *Pantoea* comprises many versatile species with different functions, like the degradation of herbicides and other toxic compounds [83]. Some *Pseudoalteromonas* strains can produce bioactive compounds [84], and Sporobolomyces is a yeast capable of pesticide degradation [60]. Under Org_C, *Turicibacter, Dinghiubacter, Planifilium, Roseibacillus*, *Piscibacillus*, and *Candida* were more abundant and were positively correlated with ammonium, indicating that the presence of ammonium could favor the presence of these genera. Previous studies also reported that *Turicibacter* are abundant in soil with manure application and a high total organic carbon [85]. Regarding to *Candida*, it is important to point out that it is a saprotrophic fungi that sometimes come from compost, and may cause an opportunistic hazard to human health [86].

Org_M-related genera were *Aciditerrimonas*, *Isoptericola*, *Nibribacter*, *Haliangium*, *Terrimicrobium*, *Funneliformis*, *Wallemia*, *Melanoleuca*, and *Filobasidium*. *Aciditerrimonas* is related to ferrous-ferric redox [87], and showed a correlation with TOC and NH_4^+. *Haliangium* is a genus in which some species are producers of *haliangicins*, known as antifungal compounds [88], or *Wallemiales*, which have been observed in organic cultivations in other studies [7], and have shown a strong negative correlation with *Alternaria* spp. and *Fusarium Oxysporum*. This suggests that an increase in this genus could favor a decrease in some phytopathogens [89], as we observed in this cropping system.

5. Practical Implications of This Study

In this study, the impacts of two types of long-term organic farming and one conventional farming system on the soil chemical properties and microbial communities are highlighted. This study highly recommends organic farming, principally the one where compost was used as an organic amendment (Org_C), where no differences were observed on crop yield with the other systems and it had a stable system with a high total organic carbon and nutrients, as well as a change in the bacterial and fungal communities. In addition to the environmental and human benefits of this type of farming system, further studies should further examine the possible mechanisms behind microbial community changes related to specific biogeochemical cycles, as well as the functional approach and inter-connection between microbial communities.

6. Conclusions

This work showed that properly long-term organic systems can contribute to maintaining high-yielding and stable crops when compared to the conventional system. In particular, it is noticeable that Org_C showed a change in bacterial community associated with an improvement in the soil carbon and nutrient content that was not found with Org_M. Analyses showed that fungal communities were more sensitive to cropping systems than bacteria because of the changes on a phylum level, while bacteria changes were more apparent on a specific taxonomy level. In addition, the LEfSe analysis revealed different microorganisms associated with each of the studied cropping systems. In particular, for the Conv system, the analysis demonstrated the presence of microorganisms associated with pesticide, while Org_C and Org_M microorganisms were associated with organic matter decomposition with different putative functions that, in some cases, could reduce soil borne pathogens.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agriculture11050445/s1, Figure S1: Rarefaction curves for the bacterial and fungal soil sequences, Table S1: Characteristics of organic amedments, Table S2: Diversity index for the bacterial and fungal community, Table S3: Relative abundance of most abundant bacterial phyla, Table S4: Relative abundance of most abundant fungal phyla, Table S5: NMDS results between bacterial community and the significant properties, Table S6: NMDS results between fungal community and the significant properties.

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Supplementary Material of Changes in Bacterial and Fungal Soil Communities in Long-Term Organic Cropping Systems

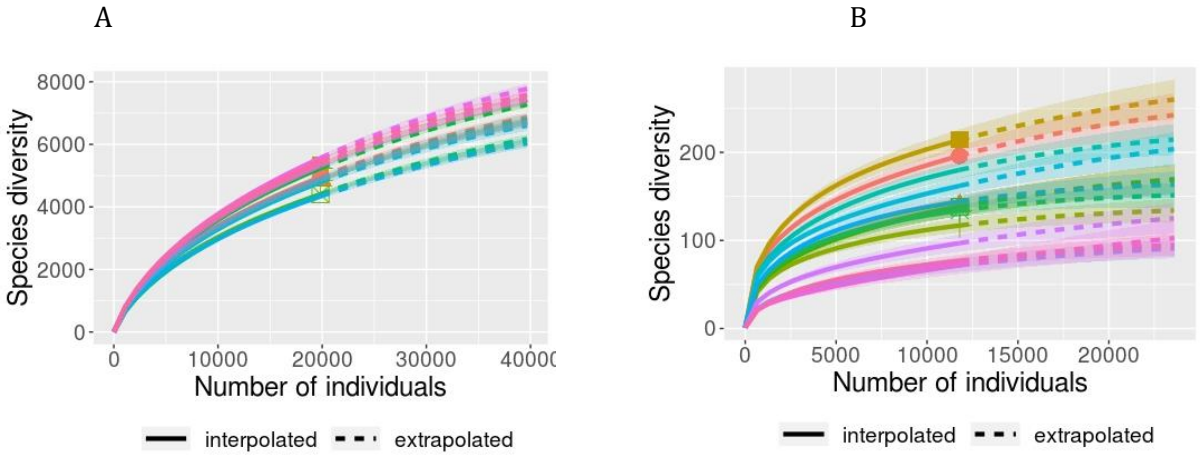


Figure S1. Rarefaction curves for the (A) bacterial (B) fungal soil sequences. The curves were obtained after getting the same depth in the samples, 19840 sequences in bacterial data and 15900 sequences in fungal data. (Species diversity = Chao1)

Table S1. Characteristics of organic amendments. (mean ±standard deviation, (n=3))

	TOC g kg^{-1} / mg L^{-1}	Total N g kg^{-1} / mg L^{-1}	C/N	Ca g kg^{-1} / mg L^{-1}	Mg g kg^{-1} / mg L^{-1}	P g kg^{-1} / mg L^{-1}	K g kg^{-1} / mg L^{-1}	Na g kg^{-1} / mg L^{-1}
Manure	101±18	3.9±0.3	24.9±3.5	185.6±25.3	8.4±1.0	0.9±0.1	17.6±3.9	1.5±0.8
Compost	110±13	8.3±0.5	13.4±1.8	161.6±13.9	10.2±0.6	1.3±0.1	36.2±6.0	3.1±1.0
Compost tea	143±19.6	21.1±6.8	7.2±1.7	59.0±12.5	48.9±7.9	12.7±3.3	203.5±29.9	246.1±34.1

Table S2. Diversity index (Chao1 and Shannon) for the bacterial and fungal community in the three cropping systems. (mean±SD (n=5)).

Bacterial				
Index	**Conv**	**Org_C**	**Org_M**	**ANOVA**
Chao1	8833.45±619.78	9718.96±665.32	8673.16±633.46	ns
Shannon	7.53±0.17	7.71±0.09	7.42±0.23	ns
Fungal				
Index	**Conv**	**Org_C**	**Org_M**	**ANOVA**
Chao1	229.52±76.17	138.24±36.25	201.55±42.45	ns
Shannon	2.84±0.60 a	1.27±0.35 b	2.56±0.41 a	**

ns = non-significant; values followed by different letters correspond to significant differences (Tukey's test); significant levels: *** p < 0.001; **, p< 0.01; *, p < 0.05; Conv, Conventional system; Org_C, Organic cultivation with manure compost and tea compost; Org_M, Organic cultivation with manure.

Table S3. Relative abundance of most abundant (>1%) bacterial phyla in the three cropping systems.

Taxonomy	Conv	Org_C	Org_M
Proteobacteria	42.03±6.04	42.11±2.55	41.03±6.66
Actinobacteria	17.78±4.12	15.00±3.36	16.40±4.74
Bacteroidetes	14.66±4.03	9.92±2.20	11.42±3.08
Gemmatimonadetes	11.50±5.31	13.63±1.73	12.87±3.23
Acidobacteria	5.7±1.39	8.07±1.74	8.07±2.23
Planctomycetes	3.35±0.53	5.05±1.11	4.59±0.95
Chloroflexi	1.85±0.13	2.6±0.20	2.41±0.64
Verrucomiota	1.65±0.53	1.89±0.28	2.11±0.41
Firmicutes	1.43±0.87	1.70±0.29	1.09±1.03

Conv, Conventional system; Org_C, Organic cultivation with manure compost and tea compost; Org_M, Organic cultivation with manure. Classified as uncultured and unknown were not shown. (Mean±SD (n=5))

Table S4. Relative abundance of most abundant (>1%) fungal phyla in the three cropping systems.

Taxonomy	Conv	Org_C	Org_M
Olpidiomycota	9.55±5.17	65.65±41.00	0.74±1.05
Basidiomycota	21.84±12.63	23.50±47.00	36.62±23.57
Glomeromycota	14.34±24.28	0.00±0.00	33.55±23.23
Ascomycota	26.32±9.09	7.78±5.53	11.43±4.67
Mortierellomycota	20.79±7.29	3.07±1.86	15.24±6.12
Mucoromycota	6.41±6.73	0.00±0.00	2.41±4.28
Chytridiomycota	0.42±0.84	0.00±0.00	0.00±0.00

Conv, Conventional system; Org_C, Organic cultivation with manure compost and tea compost; Org_M, Organic cultivation with manure. Classified as uncultured and unknown were not shown. (mean±SD (n=5))

Table S5. NMDS results between bacterial community and the significant soil properties.

	NMDS1	NMDS2	r2	Pr(>r)
pH	0.31911	0.94772	0.0291	0.845
TOC	0.61792	-0.78624	0.6047	0.005 **
POC	-0.62080	0.78397	0.1902	0.291
TN	0.69486	-0.71915	0.5480	0.011 *
Mg	0.65220	-0.75805	0.4482	0.031 *
K	0.68028	-0.73295	0.2577	0.144
NH4	0.44548	-0.89529	0.6670	0.001 ***
NO3	-0.23042	0.97309	0.1870	0.290
Fe	0.62790	-0.77829	0.2457	0.188
Mn	-0.05603	-0.99843	0.1131	0.469
B	0.50667	-0.86214	0.4486	0.038 *
TP	-0.72397	0.68983	0.2281	0.204
ALT	0.06553	-0.99785	0.0951	0.552
FOX	0.03293	-0.99946	0.3318	0.114

Significant levels: *** p < 0.001; **, p< 0.01; *, p < 0.05; TOC, Total Organic Carbon; TN, Total Nitrogen; POC, Particulate Organic Carbon; Mg, Available Mg; K, available K; Fe, available; Mn, available Mn; B, available B; TP, Total Pesticides; ALT, *Alternaria spp*.; FOX, *Fusarium Oxysporum.*

Table S6. NMDS results between fungal community and the significant soil properties.

	NMDS1	NMDS2	r2	Pr(>r)
pH	-0.92374	0.38302	0.0036	0.979
TOC	-0.64078	-0.76772	0.0253	0.852
POC	-0.99309	-0.11735	0.0057	0.975
TN	-0.87791	0.47883	0.0228	0.886
Mg	-0.96714	-0.25426	0.1068	0.596
K	-0.85364	0.52087	0.2280	0.271
NH4	-0.22551	0.97424	0.1201	0.516
NO3	0.16120	-0.98692	0.1488	0.448
Fe	-0.34307	-0.93931	0.1793	0.394
Mn	0.30863	-0.95118	0.3478	0.124
B	0.01558	-0.99988	0.0219	0.888
TP	0.61561	-0.78805	0.3771	0.068
ALT	0.28907	-0.95731	0.2485	0.248
FOX	-0.19597	-0.98061	0.2689	0.212

Significant levels: *** $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; TOC, Total Organic Carbon; TN, Total Nitrogen; POC, Particulate Organic Carbon; Mg, available Mg; K, available K; Fe, available; Mn, available Mn; B, available B; TP, Total Pesticides; ALT, *Alternaria spp*.; FOX, *Fusarium Oxysporum.*

Long-Term Compost Amendment Changes Interactions and Specialization in the Soil Bacterial Community, Increasing the Presence of Beneficial N-Cycling Genes in the Soil

Abstract

Within sustainable agriculture, organic farming is more environmentally friendly than conventional farming while producing higher quality crops and similar yields. Soil organic matter content plays an important role in soil fertility, maintaining soil functions and reducing erosion. Manure and principally compost are real alternatives for incorporating organic matter and nutrients into agricultural soils. The complex interaction among different microbial species through the flow of energy, matter, and information forms large, complex ecological networks that it is essential to understand the underlying mechanisms to produce higher yields and maintain or even increase soil quality through sustainable agricultural practices. For organic cropping systems, nitrogen has been identified as the main yield-limiting nutrient so in-depth knowledge of its transformation and fixation in the soil could help to understand which soil microorganisms are better adapted for a specific crop management.

In this work, soil bacterial community measured by 16S rDNA amplicons from two long-term organic farming (organic cultivation with manure compost and compost tea (Org_C) and organic cultivation with manure (Org_M)) and a conventional system (Conv) are studied to explore the effect of farming systems on phylogenetic molecular ecological networks (pMENs), and to extract co-occurrence patterns and to identify microorganisms with key topological roles involved in such a complex networking. To predict soil microbial functionalities on organic farming compared to conventional system by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt), principally the functional genes associated to nitrogen cycle. The relationships among bacterial modules and physicochemical soil properties are evaluated by Molecular Ecological Network Analysis (MENA). First, a Welch's test followed by a Games-Howell post-hoc test are employed to compare topological networkproperties under different cropping systems. Afterward, the predicted genes belonging to nitrogen cycle are analysed using Analysis of Variance (ANOVA) with bootstrap resampling (with 1000 replicates) or Kruskal-Wallis test as nonparametric alternative whereas changes in the general functionality are analysed through Permutational Analysis of Variance (PERMANOVA) using Bray-Curtis distance. To test the significant relationships between soil properties and microorganisms, mantel test is performed.

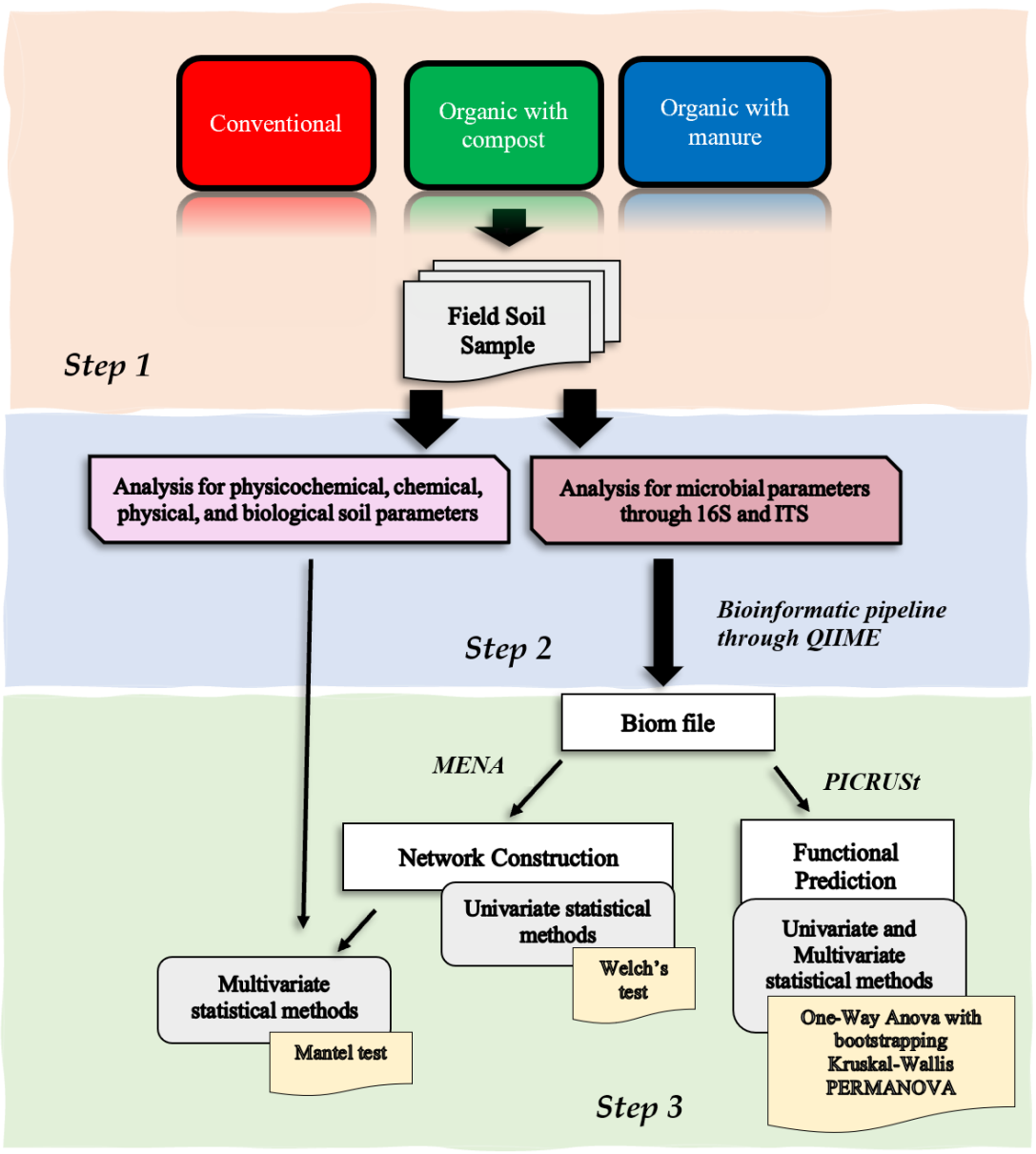
Results show that the bacterial community in the compost amended soil (Org_C) shows the higher connectivity, clustering coefficient, modulation, number of modules, negative connections, and generalists than the other two cropping systems. The incorporation of organic matter into the soil promoted some specific generalists, including Firmicutes (*Bacillus*) and Verrucomicrobia in Org_M; and Planctomycetes, Chloroflexi and NKB19 in Org_C, considered some of them plant-beneficial microorganisms. The roles of some nodes shifted in the three networks systems indicating change in the ecological roles of key bacteria. Nodes belonging to Proteobacteria and Actinobacteria, which are considered generalist in the Conv network, are considered specialist in the Org_C and Org_M networks. Both organic cropping systems show significant correlation between TOC and TN with microorganisms, although TN has a negative correlation in Org_M whereas it is positive in Org_C, instead of TOC has a positive and negative correlation in Org_M whereas it is only positive in Org_C.

Analysis of the most abundant metabolism pathway genes reveal that, in general, the relative abundance of N_2-fixing functional genes (*nifH*, *nifD* and *nifK*) are significantly higher in both organic treatments than in convention; in Org_C is also significantly higher than in Org_M. Predicted denitrification genes, such as the denitrifying nitrous oxide reductase gene (*nosZ*) or nitrate reductase (*narG*), do not show any significant differences between the three cropping systems. Nitric oxide reductase (*norB*) and nitrite reductase (*nirK*) show significantly higher predicted abundance in Org_C and Conv than in Org_M, while ammonium-forming nitrite reductase (*nrfA*) and nitrate reductase (*narH*) show higher values in Org_C than in

Org_M. In the nitrification process, the ammonia oxidation-predicted genes (*amoA*/*amoB* and *amoC*) are significantly increased in Conv and Org_C compared to Org_M.

Relevant contribution reached with this paper is that cropping systems affect microbial structure, its relationships, and their potential functionality and that it can be carried using non common algorithms conducted by bioinformatic pipelines such a MENA or PICRUSt. Long-term compost addition (Org_C) not only improves soil quality and properties, but it also notably alters bacterial community making more versatile and resistant to changes due to its high modularity, being able to help protect and feed the crops; it would imply higher soil quality, because it is more resilience to changes. In addition, the high number of niches and available nutrients seems to enhance the growth of specific microorganisms capable of metabolizing nitrogen, increasing its nitrogen-fixing potential, decreasing N_2O emissions and increasing the carbon-sequestration potential by autotrophic microorganisms compared to the other cropping systems. This implies the importance of using a stable organic amendment as compost and the use of adequate tools to study the response of the bacterial community to long-term cropping systems.

Workflow


Conventional
Organic with compost
Organic with manure
Field Soil Sample
Step 1
Analysis for physicochemical, chemical, physical, and biological soil parameters
Analysis for microbial parameters through 16S and ITS
Bioinformatic pipeline through QIIME
Step 2
Biom file
MENA
PICRUSt
Network Construction
Univariate statistical methods
Welch's test
Functional Prediction
Univariate and Multivariate statistical methods
One-Way Anova with bootstrapping
Kruskal-Wallis
PERMANOVA
Multivariate statistical methods
Mantel test
Step 3

agronomy

MDPI

Article

Long-Term Compost Amendment Changes Interactions and Specialization in the Soil Bacterial Community, Increasing the Presence of Beneficial N-Cycling Genes in the Soil

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Abstract: Significant differences in the microbial community and diversity in soil have been observed due to organic farming, but little research has been performed for exploring microbial functionality and the co-occurrence of patterns among microbial taxa. In this work, we study soil 16S rDNA amplicons from two long-term organic farming systems (Org_C and Org_M) and a conventional system (Conv) to decipher the differences in microbial interaction and network organization and to predict functional genes (principally related to the N cycle). In general, the network organizations were different in all cropping systems due to agricultural management. Org_C showed the highest negative interactions and modularity and the most altered bacterial niches and interactions, which led to an increase in generalist species that stabilize the bacterial community and improve the response of the soil to adverse conditions. These changes altered the predicted functionality of the bacterial community; Org_C showed higher referred numbers of nitrogen fixation genes, a decrease in the N_2O emission genes and could favor the uptake of environmental CO_2. Thus, long-term compost amendment application has significant benefits for the farmer and the environment, since prolonged application can reduce the use of fertilizers and pesticides and could create a more stable soil, which could resist the effects of climate change.

Keywords: co-occurrence; conventional farming; manure; organic farming; PICRUSt; soil bacteria

1. Introduction

In recent decades, the concept of sustainable agriculture has been gaining ground. Sustainable agriculture is an integrated system in which plant production practices have a site-specific application that has long-term benefits [1]. Sustainable agriculture not only satisfies human food and fiber needs, but also enhances environmental and natural resource quality [2]. Crop rotation, intercropping and organic fertilizers are common sustainable practices across a diverse array of agroecosystems. These practices can break cycles of disease and pests, improve soil fertility, suppress weeds and improve food and nutritional security [3,4].

Within sustainable agriculture, organic farming is perceived to be more environmentally friendly than conventional farming while producing higher quality crops and similar yields [5,6]. Soil organic matter content plays an important role in soil fertility, maintaining soil functions and reducing erosion. However, its build-up is a very slow process, whereas

its decline is relatively fast [7]. Soil organic matter usually depends on the input of organic material, and it contributes to the improvement of physicochemical, chemical and biological properties of soils. Compost is one of the best alternatives for incorporating organic matter and nutrients into the soil; it is considered a cheap organic amendment that is agronomically advantageous and environmentally safe, and which stimulates soil microbial activity and crop growth [5,8].

Soil microorganisms maintain soil health and are crucial for crop production in agricultural systems. They play an essential role in the soil structure as well as in decomposing organic matter, degrading contaminants, suppressing soil-borne diseases [9] and fertilizing the soil [10,11]. The complex interaction among different microbial species through the flow of energy, matter and information forms large, complex ecological networks; it is essential to understand the underlying mechanisms in order to fully understand the soil microbiota [12]. Determining microbial network structures and their relationships to environmental changes and metabolic processes in microbial communities is a significant challenge in agricultural soils [13].

Microbial metabolism involves a large set of functional genes and biochemical pathways that power biogeochemical cycling in soil. Nitrogen has been identified as the main yield-limiting nutrient for organic cropping systems [14], so in-depth knowledge of its transformation and fixation in the soil could help to understand which soil microorganisms are best adapted to the soil for a specific crop [15,16].

A good approach for studying the potential functionality of microbial communitieshas recently been defined. This approach consists of predicting functionality from 16s rRNA gene sequence data based on Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt), which uses an extended ancestral-state construction as a predictor [17]. This procedure is followed by predicting the functional genes associ-ated with nitrogen metabolism based on the Kyoto Encyclopedia of Genes and Genomes database (KEGG). This method has gained popularity in recent years in ecology [18,19] and agriculture [20,21], but the limitations of a metagenomic technique must be takeninto account. These limitations are mainly due to the short fragment length and potential inaccuracies in the prediction process, so we should keep these factors in mind as we consider potential functionality.

To analyze the interactions among different microbial communities, a network-based bioinformatics approach has been used. This approach is based on high-throughput metagenomics sequencing data, where the network is a representation of various biological interactions, e.g., predation, competition and mutualism in soil in which species (nodes) are connected by pairwise interactions (edges) [22,23].

Future agriculture needs to focus on measures that improve soil biological functions for appropriate soil health management [24]. Functional traits and phylogenetic network analyses are valuable ecological markers for understanding microbial community assembly, and they help elucidate how natural communities and their functions respond to environmental and soil management changes [25–27].

We hypothesized that bacterial communities become stable over time under different cropping systems and that bacterial communities under long-term organic farming systems will be more diverse and will show a greater capacity to adapt to external agents. The objectives of this work were: (i) to explore the effect of farming system on network interactions among different phylogenetic groups by phylogenetic molecular ecological networks (pMENs); (ii) to study the predicted functions of an organic farming system compared to a conventional system, principally the functional genes associated with nitrogen cycle; and (iii) evaluate the relationships among bacterial modules and physicochemical soil properties.

2. Materials and Methods

2.1. Experimental Design and Sampling

The soil for the study was Haplic Calcisol (loamic, hypercalcic) IUSS [28], located in Campo de Cartagena in Murcia, southeastern Spain. Since the early 1990s, fifteen plots have been used for vegetable cultivation with three different cropping systems (five plots for each): (1) a conventional system (Conv); (2) an organic system with a yearly addition of compost and compost tea (Org_C); and (3) an organic system with a yearly addition of sheep manure (Org_M). More information can be found in Table S1. The characteristics of the sheep manure, compost and compost tea were previously described in Cuartero et al. [29]. The sampling was carried out in February 2018 after the harvest of a leaf cabbage crop (Brassica olearacea var. sabellica) grown during the winter season. One composite sample derived from 10 subsamples (0–10 cm depth) was collected with an auger from each plot.

Samples were taken to the lab immediately and separated into two aliquots. The soil for biological analysis was sieved at <2 mm and stored at −20 °C, and the soil for physicochemical and chemical analyses was sieved at <2 mm and kept at 4 °C. Soil properties were measured according to Cuartero et al. [29].

2.2. Soil Properties, DNA Extraction, Sequencing, Data Processing and Function Prediction

Total organic carbon (TOC), pH, electrical conductivity (EC), total nitrogen (TN), $NH4^+$, available P, Mg, Na and Ca were measured according to Cuartero et al. [29]. Soil DNA extraction, bacterial community analysis (by next generation sequencing of bacterial 16S hypervariable regions) and data processing were performed according to Cuartero et al. [29] using the Greengenes database. The sequences are available at the European Nucleotide Archive (ENA) with the study accession code PRJEB38121.

The metagenomes were predicted from the OTU table using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) [17]. The OTUs were normalized by dividing each OTU by the predicted 16S copy number abundance, and the functional genes were identified based on Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways [30].

2.3. Construction and Analysis of the Microbial Network

Network analyses were performed to discern co-occurrence patterns of the soil microorganisms by constructing phylogenetic molecular ecological networks (pMENs) through the open-access molecular ecological network analysis pipeline (http://ieg4.rccc.ou.edu/mena/, accessed on 17 January 2022) [12,13,31]. Network construction was based on relative abundance in the soil samples for each cropping system. The relative abundances of OTUs were transformed into log matrixes, and a Pearson correlation matrix was estimated. A reliable similarity threshold (St) for the correlation matrix based on the χ 2-test with Poisson distribution was automatically identified according to a Random Matrix Theory (RMT)-based approach prior to the network construction. The adjacency matrix was derived only from OTUs with similarity values above an optimal St. These OTUs were represented as nodes, and their pairwise interactions were represented as edges. For each of the resulting pMENs, 100 randomly rewired networks were generated, keeping the network size and number of links. Welch's t-test followed by a Games—Howell post-hoc test were performed to compare topological network properties under different cultivation systems from the standard deviations reported from their respective random networks. Network modules were detected using the fast greedy modularity optimization method. Eigengene network analysis was carried out by performing singular value decomposition (SVD) in order to summarize each module with a single representative abundance pro- file, known as the module eigengene. Small modules with fewer than five nodes were not used. Moreover, hierarchical cluster trees and heatmaps were derived to display the module eigengenes of each module, higher-order organization and correlations between modules. Identification of key module members (MMs) was based on threshold values of Zi (within-module connectivity) of 2.5 and Pi (among-module connectivity) of 0.62 accord-

ing to Guimera and Nunes Amaral [32] and Olesen et al. [33]. OTU roles can be thereby categorized into peripherals (Zi < 2.5; Pi < 0.62), connectors (Zi < 2.5; Pi ≥ 0.62), module hubs (Zi ≥ 2.5; Pi < 0.62) and network hubs (Zi ≥ 2.5; Pi ≥ 0.62). Furthermore, Mantel tests were performed to detect relationships under the three cropping systems between network connectivity, soil properties and metabolic functionalities. OTU significance was calculated previously. The networks were visualized using Cytoscape software version 3.5.1 [34] and the ggplot2 package [35].

2.4. Statistical Analysis

To evaluate the effect of cultivation systems on OTU variations, permutational multivariate analysis of variance (PERMANOVA) was conducted using the 'betadisper' and 'adonis' functions with 999 permutations from the vegan package version 2.5-7 [36]. Furthermore, functional profile assignments from PICRUSt were also tested with R version 4.0 [37]. Normality and homogeneity of variance assumptions were assayed by Shapiro—Wilk and Bartlett's tests. Mean comparisons were performed with one-way analysis of variance (ANOVA) followed by post-hoc tests, Tukey's honestly significant difference (HSD) for all-pair comparisons and Dunnett's comparisons for the control system. In cases in which homoscedasticity was not met, Welch's t-test was performed using the 'pairwise.*t*.test' function with Bonferroni—Holm correction for multiple comparisons. The robustness ofthe estimations was checked by the bootstrapping approach using 1000 replicates. When data did not fit a normal distribution, non-parametric Kruskal—Wallis tests were per- formed, and if the assayed data were significant, a multiple comparison *Z*-values test was performed using the 'dunnTest' function with Benjamini—Hochberg corrections in the FSA package version 0.8.30 [38].

3. Results

3.1. Network Analysis

After data preprocessing, 539 OTUs remained in both the Conv and Org_C data sets for network construction, and 439 OTUs remained in the Org_M data set. Optimal similarity thresholds for the correlation matrixes obtained were identical (0.97) for the three soil microbial communities. Applying such a cut-off, two networks of similar size, nodes and links were constructed for Conv and Org_C, and another one of a smaller size was constructed for Org_M (Table 1). In addition, network connectivity distribution curves fitted well with the power law model (R2 varied from 0.75 from 0.83).

Table 1. Topological properties of the empirical pMENs of microbial communities of the three cropping systems and their associated random pMENs.

Treatment	Empirical Networks										Random Networks		
	No. of Original OTUs	Similarity Threshold St	Network Size	R Square of Power-Law	Avg Con-nectivity	Node	Edge	Average Path Distance (GD)	Avg Clustering Coefficient	Modularity (No. of Modules)	Avg Path Distance ±SD	Avg Clustering Coefficient ± SD	Avg Modularity ± SD
Conv	539	0.97	453	0.83	6.04	404	1220	6.821 b	0.368 b	0.646 (32) c	3.430 ± 0.031	0.037 ± 0.004	0.369 ± 0.005
Org_C	539	0.97	452	0.80	6.80	400	1360	6.896 b	0.395 a	0.698 (36) b	3.300 ± 0.031	0.044 ± 0.005	0.337 ± 0.005
Org_M	439	0.97	396	0.75	4.08	357	729	8.262 a	0.355 c	0.824 (29) a	4.176 ± 0.038	0.014 ± 0.04	0.497 ± 0.008

Values followed by different letters represent significant differences between cropping systems by Games—Howell's post-hoc test: Conv, conventional system; Org_C, organic cultivation with sheep manure compost and compost tea; Org_M, organic cultivation with sheep manure.

Data revealed modularity values higher than 0.4. The highest connectivity and clustering coefficient was found for the Org_C network, followed by the Conv and Org_M networks. The average path distance and modularity were lower for Conv, followed by the Org_C and Org_M networks (Table 1). In addition, the average path distance and modularity of the networks were larger than their respective random networks (Table 1).

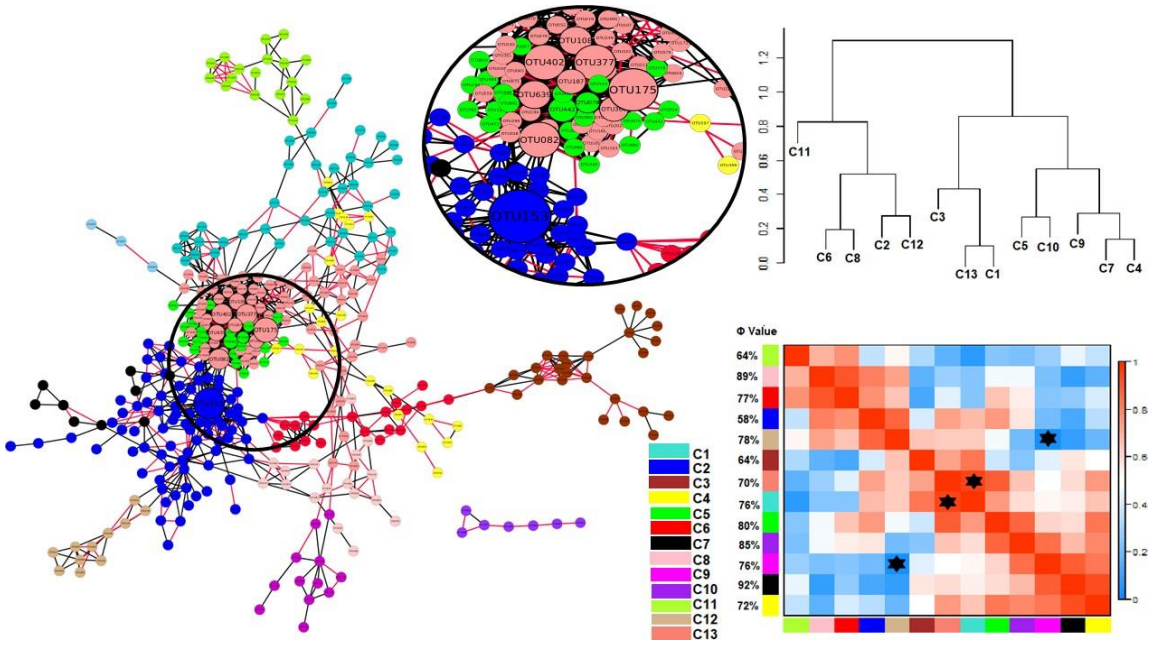
Overall network indices for the identified pMENs under the distinct cultivation sys- tems reported significant differences. We found that the Org_C network was composed of 400 nodes (OTUs) linked by 1360 edges (789 positive edges and 571 negative edges); Conv

by 404 nodes and 1220 edges (875 positive edges and 345 negative edges); and Org_M by 357 nodes and 729 edges (453 positive edges and 276 negative edges). The Org_C network showed 15 major modules (modules with more than 5 nodes), followed by Conv with 13 modules and Org_M with 12 modules (Figure 1).

All of the nodes included in the major modules had significant ($p < 0.05$) module memberships (MMs), as shown by module eigengene analysis. A total of 1015 significant MMs were observed, of which 703 were shared between the three cropping systems, accounting for 59% of the Org_C network, 65% of the Org_M network and 57% of the Conv network. In general, Actinobacteria and Proteobacteria were the dominant phyla in the three networks, but Firmicutes, Bacteriodetes, Acidobacteria and Verrucomicrobia were widely distributed as well (Table S2).

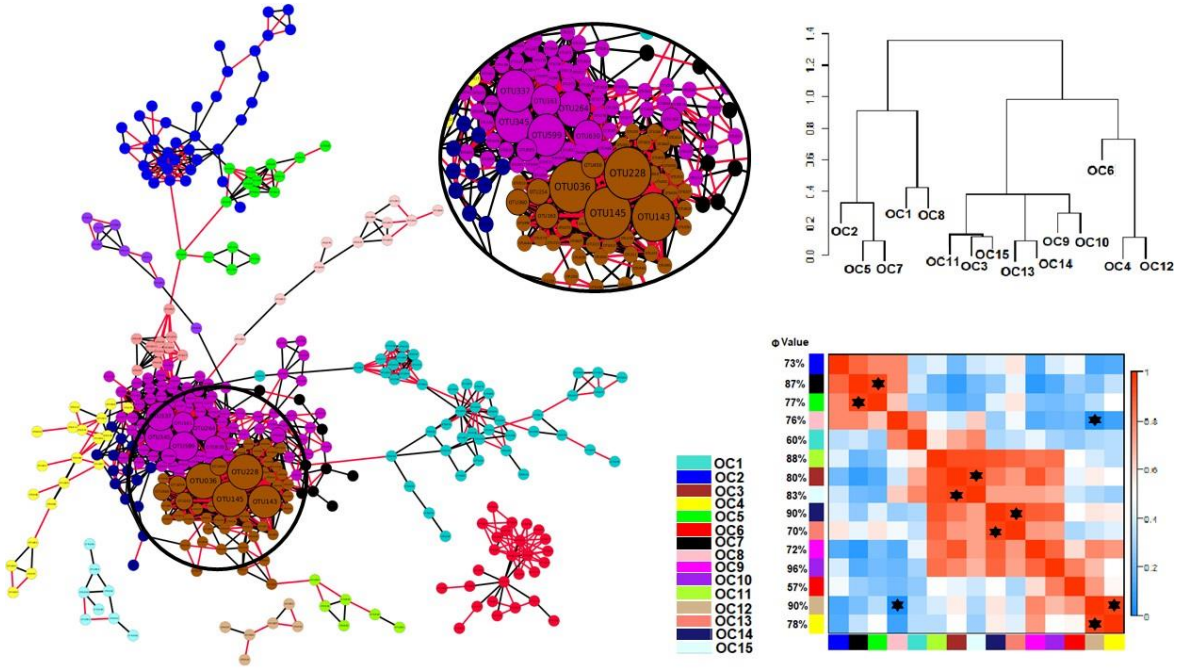
Moreover, eigengene analysis, based on the clustering dendrogram and heat map, revealed differences in higher-order organization between the networks (Figure 1). Module eigengenes explained 57–96%, 58–92% and 59–89% of the variations of relative abundance across different samples in the Org_C, Conv and Org_M networks, respectively (Figure 1). Eigengenes from modules showed significantly higher correlations among modules in the Org_C (OC5-OC7; OC3-OC15; OC14-OC13; OC4-OC12 and OC12-OC13) and Conv (C1-C13 and C9-C12) networks, whereas Org_M did not show significant correlations among modules (Figure 1).

The eight OTUs with the highest abundance (436; 605; 110; 003; 324; 438; 486 and 174) and their nearest neighbors were selected for study of the connection variations in a subnetwork. The Org_C subnetwork had the highest number of connections (50 nodes and 130 edges), followed by Org_M (47 nodes and 111 edges) and Conv (43 nodes and 91 edges) (Figure 2). The most abundant OTUs showed more direct connections and interactions in Org_C, with 46 nodes and 46 edges (28 positives and 18 negatives), than in Org_M, with 40 nodes and 40 edges (37 positive and 3 negative), or Conv, with 35 nodes and 35 edges (12 positives and 23 negatives) (Table S3).



(**A**)

Figure 1. *Cont.*



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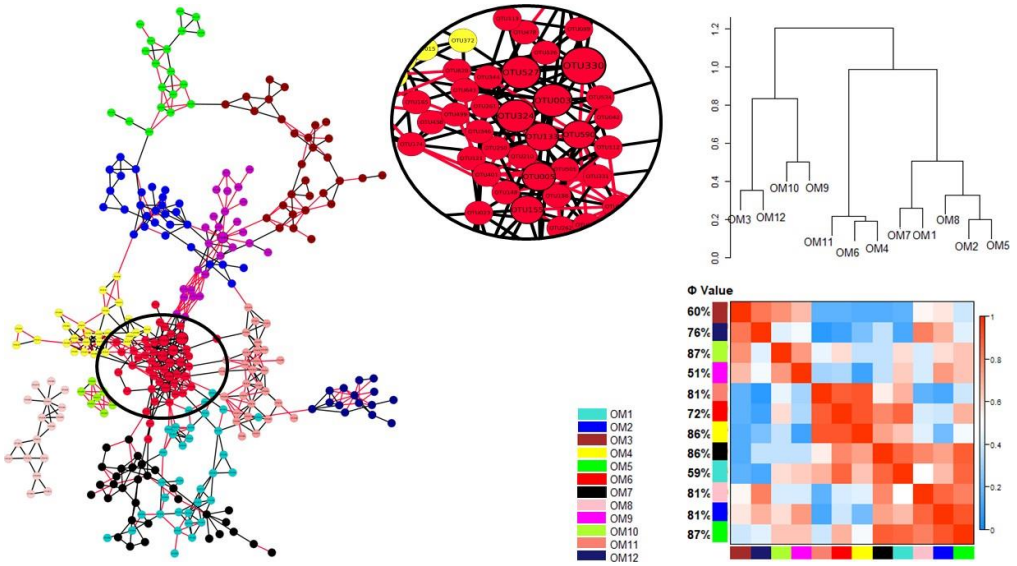


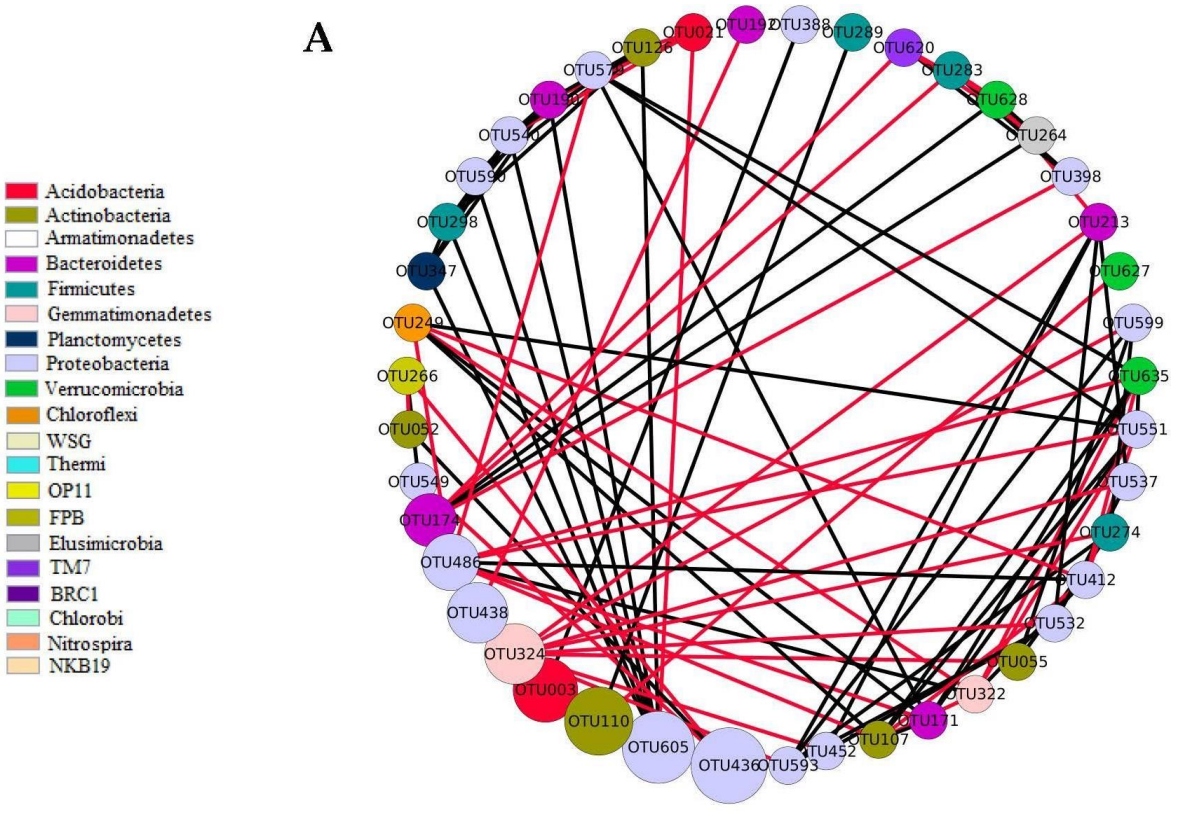
(**C**)

Figure 1. Networks of (**A**) the conventional cropping system (Conv) and both organic cropping systems, (**B**) Org_C and (**C**) Org_M, based on OTU profiles. On the top right a hierarchical clustering is shown based on Pearson correlations among module eigengenes. Below the clustering, a heatmap shows the coefficient values. The color red means higher correlation, while blue signifies lower correlation. Modules smaller than five nodes were excluded from eigengene analysis and are not displayed. Modules larger than five nodes are labelled with different colors in the network. The size of the circle is directly proportional to the number of edges in the module, and a large circle implies a higher number of connections in the node. Red and black lines represent negative and positive edges, respectively. A black star on the heatmap (★) indicates a significant ($p < 0.05$) correlation among modules: Conv, conventional cropping system; Org_C, organic cropping system with compost and compost tea; Org_M, organic cropping system with manure.

3.2. The Generalist Presence in Networks

A Zi-Pi plot was constructed to illustrate the topological roles of individual network nodes (Figure 3). The majority of the OTUs (>98%) observed in the three pMENs were categorized as peripherals (representing specialist nodes from an ecological perspective) with most of their edges inside their own modules (71.6% for Conv, 75.3% for Org_C and 82.3% for Org_M) (Figure 3). Few module hubs (generalists) were present (1% for Conv, 1.5% for Org_C and 1.7% for Org_M). In addition, a total of two nodes (0.5%) were connectors (generalists) for the Conv and Org_C networks, whereas none were identified for the Org_M network. No network hubs (supergeneralists) were observed in any of the three networks.

Six module hubs belonging to Actinobacteria (*Agromyces* and Solirubrobacterales), Proteobacteria (*Arenimonas*, *Ramlibacter* and *Geobacter*), Planctomycetes, Chloroflexi and NKB19 were observed in the Org_C network. Five module hubs belonging to Actinobacteria, Protobacteria, Firmicutes (*Bacillus*) and Verrucomicrobia were found in the Org_M network. In the Conv network, four module hubs were found belonging to Actinobacteria (*Nocardioides* and *Rubrobacter*) and Proteobacteria (*Methylotenera* and *Sphingopyxis*) (Table S4). Connectors in the Org_C network (OTU461 and OTU508) belonged to Proteobacteria, and those in the Conv network (OTU085 and OTU442) belonged to Actinobacteria and Proteobacteria (Table S4). Interestingly, some of the nodes inverted their topological function, serving as a generalist in one network and a specialist in another (Table S4).



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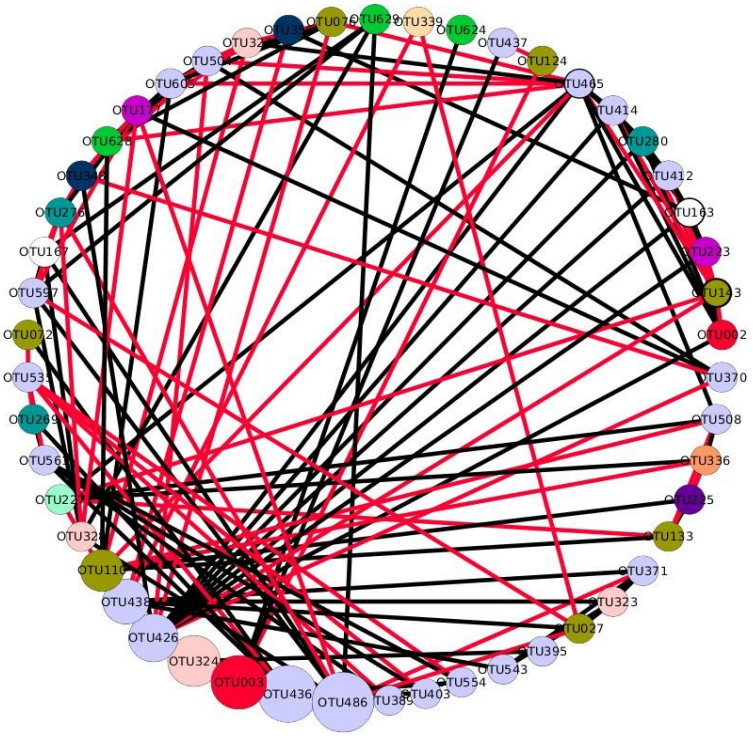

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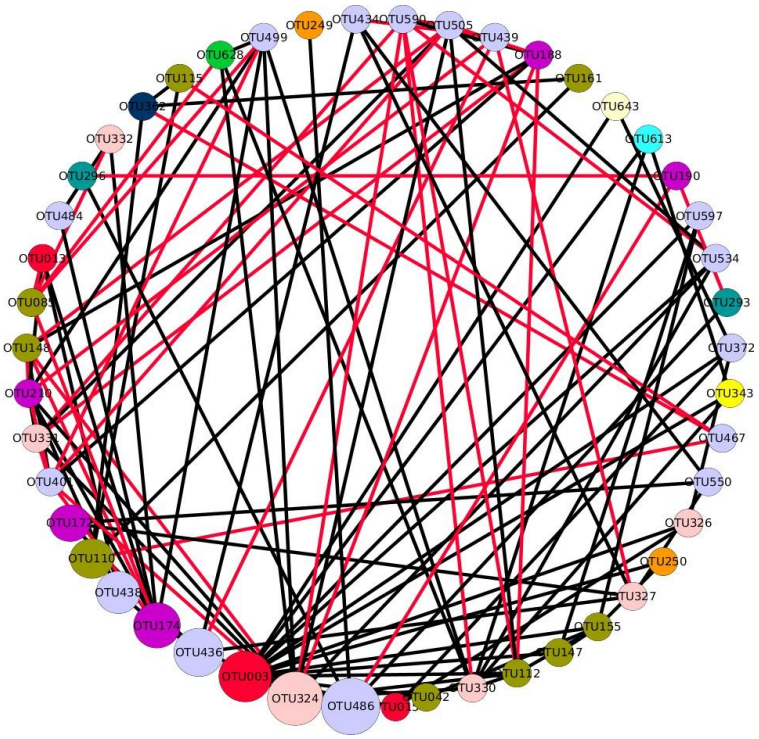

Figure 2. Subnetwork of the eight most abundant OTUs and their first neighbor nodes in the (**A**) conventional cropping system (Conv) and both organic cropping systems, (**B**) Org_C and (**C**) Org_M. Each circle represents an OTU, and its color represents a phylum. The size of the circle corresponds to the OTU's abundance; OTUs are arranged so that they neighbor OTUs of similar size. Red and black lines represent negative and positive edges.

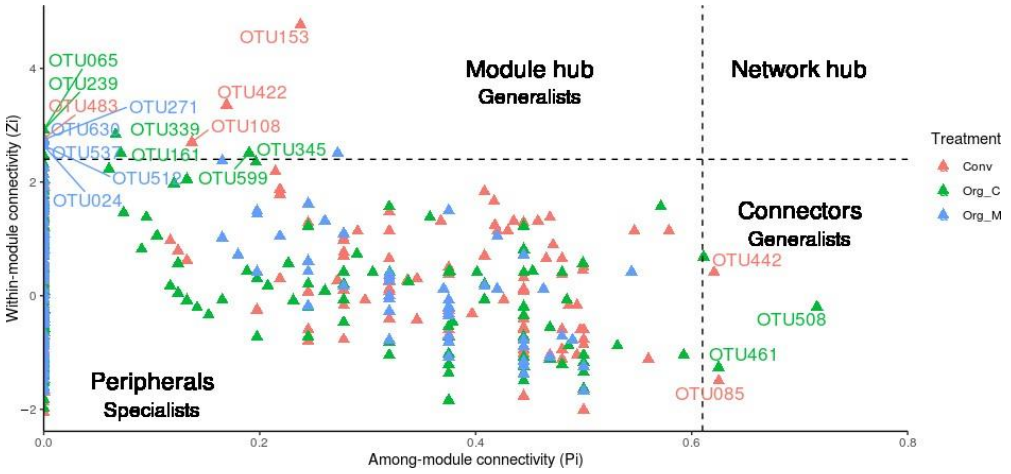

Figure 3. Zi-Pi plot showing the distribution of OTUs according to their topological roles. Each color represents the different cropping system nodes from the networks: Conv, conventional cropping system; Org_C, organic cropping system with compost and compost tea; Org_M, organic cropping system with manure; OTU065, OTU524, OTU161, OTU153, OTU108, OTU085, Actinobacteria; OTU239, Chloroflexi; OTU483, OTU537, OTU512, OTU422, OTU599, OTU442, OTU508, OTU461, Proteobacteria; OTU630, Verrucomicrobia; OTU271, Firmicutes; OTU339, NKB19, OTU345, Planctomycetes.

3.3. Predictive Functional Community

Closed-reference OTU picking resulted in 5.497 OTUs, which were classified into 6.909 predictive functional categories (Table S5). The majority (≈60%) of the functional genes were assigned to metabolism, followed by genetic information processing (≈19%), environmental information processing (≈15%) and cellular processes and organismal systems (0–5%). In general, pathways related to the following functional categories were higher in Org_C than in Conv and Org_M: carbon fixation, nitrogen metabolism, amino acid metabolism/enzymes, lipid metabolism, bacterial toxins, biosynthesis and the biodegradation of secondary metabolites, phosphotransferase system PTS, the signal transduction mechanism, tetracycline biosynthesis, toluene degradation, sulfur metabolism, phenylalanine, tyrosine and tryptophan biosynthesis (Table S5). In Org_C, benzoate degradation, DNA-replication proteins and carbon-fixation pathways in prokaryotes showed the highest values (Table S5).

3.4. Predictive Nitrogen Functional Community

Analysis of the most abundant N metabolism pathway genes revealed that the relative abundance of N_2-fixing functional genes (*nifH*, *nifD* and *nifK*) was significantly higher in Org_C than in Org_M, while no significant difference was observed with Conv, with the exception of the *nifK* gene, which showed higher values in Org_C (Table 2). Predicted denitrification genes, such as the denitrifying nitrous oxide reductase gene (*nosZ*) or nitrate reductase (*narG*), did not show any significant differences between the three cropping systems, although Org_C did have higher values (Table 2). Nitric oxide reductase (*norB*) and nitrite reductase (*nirK*) showed significantly higher predicted abundance in Org_C and Conv than in Org_M, while ammonium-forming nitrite reductase (*nrfA*) and nitrate reductase (*narH*) showed higher values in Org_C than in Org_M (Table 2). In the nitrification process, the ammonia oxidation-predicted genes (*amoA*/*amoB* and *amoC*) were significantly increased in Conv and Org_C compared to Org_M, while the nitrification functional- predicted gene hydroxylamine oxidoreductase (*Hao*) was increased significantly in Org_C compared to Org_M and Conv (Table 2).

Table 2. Predicted N gene count for the most abundant N cycling genes detected in three cropping systems.

	Gene	Conv	Org_C	Org_M	ANOVA	Kruskal—Wallis
N-fixation	*nifH*	0.42 ± 0.06 ab	0.47 ± 0.07 a	0.32 ± 0.10 b	*	-
	nifD	0.43 ± 0.07 ab	0.52 ± 0.06 a	0.36 ± 0.06 b	**	-
	nifK	0.43 ± 0.03 b	0.59 ± 0.13 a *	0.28 ± 0.05 c *	***	-
Denitrification	*narG*	0.38 ± 0.06	0.49 ± 0.11	0.36 ± 0.14	ns	-
	narH	0.38 ± 0.05 b	0.49 ± 0.05 a	0.36 ± 0.04 b	-	**
	nrfA	0.09 ± 0.00 ab	0.15 ± 0.07 a	0.07 ± 0.02 b	*	-
	nirK	0.35 ± 0.08 a	0.32 ± 0.09 a	0.19 ± 0.05 b *	*	-
	norB	0.48 ± 0.06 a	0.43 ± 0.05 a	0.29 ± 0.04 b ***	***	-
	nosZ	0.20 ± 0.03	0.25 ± 0.07	0.16 ± 0.04	ns	-
Nitrification	*amoC*	0.01 ± 0.00 a	0.01 ± 0.00 a	0.00 ± 0.00 b	-	***
	amoB	0.01 ± 0.00 a	0.01 ± 0.00 a	0.00 ± 0.00 b	-	***
	amoA	0.01 ± 0.00 a	0.01 ± 0.00 a	0.00 ± 0.00 b	-	***
	hao	0.08 ± 0.01 b	0.13 ± 0.01 a	0.08 ± 0.01 b	-	**

Values (mean ± sd; n = 5) are expressed with an e-value of 1×10^{-2}. In each cropping system, the mean value followed by *, ** or *** represents significant differences with respect to the conventional cropping system by Dunnett's test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$); missing asterisks denote non-significant differences. Different letters represent significant differences between cropping systems by Tukey's test or Dunn's Kruskal—Wallis multiple comparison test; Conv, conventional system; Org_C, organic cultivation with sheep manure compost and compost tea; Org_M, organic cultivation with sheep manure.

3.5. Soil Properties

Several chemical properties of the soils studied are shown in Table 3. Org_C soils showed the highest values of TN, NH_4^+ and TOC as well as some minerals, such as available Mg, K, Na and Ca. Org_M, on the other hand, showed the highest pH value. Available P was the highest in Conv, followed by Org_C and Org_M.

Table 3. Soil properties in the three cropping systems.

Soil Properties	Cropping System				
	Conv	Org_C	Org_M	ANOVA	Kruskal—Wallis
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	**	-
p (mg kg^{-1})	20.15 ± 5.24	14.65 ± 7.71	14.33 ± 7.48	ns	-
NH_4^+ (mg kg^{-1})	0.10 ± 0.23 b	1.33 ± 0.15 b	0.00 ± 0.00 a	-	**
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	0.85 ± 0.17	0.78 ± 0.06	*	-
Na (cmol kg^{-1})	2.12 ± 0.32	2.19 ± 0.86	1.64 ± 0.23	ns	-
Ca (cmol kg^{-1})	8.44 ± 0.83	10.03 ± 2.40	7.19 ± 1.49	-	ns

Values (mean ± sd; n = 5) followed by different lowercase 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 (-) symbol indicates the test did not proceed; significant levels: ** $p < 0.01$; * $p < 0.05$. Conv, conventional system; Org_C, organic cultivation with sheep manure compost and compost tea; Org_M, organic cultivation with sheep manure. TN, total nitrogen; TOC, total organic carbon; Mg, K, P, Na and Ca; available Mg,K, P, Na and Ca, respectively; EC, electrical conductivity.

3.6. Module and Node Correlations with Soil Properties

The heat-map representation of correlations among modules from pMENs and soil variables (Figure 4) showed that Org_C had the highest significant correlation ($p < 0.05$) among modules and soil properties, with 17 significant correlations (11 positive), followed by Org_M, with 11 significant correlations (2 positive), and Conv, with 5 significant corre- lations (3 positive) (Figure 4). TOC and TN only showed significant correlations within

organic cropping systems (Org_C and Org_M) (Figure 4). A Mantel test showed that soil properties have significant effects on the microbial communities in each cropping system, whereas they only affect functionality in the Conv cropping system (Table S6).

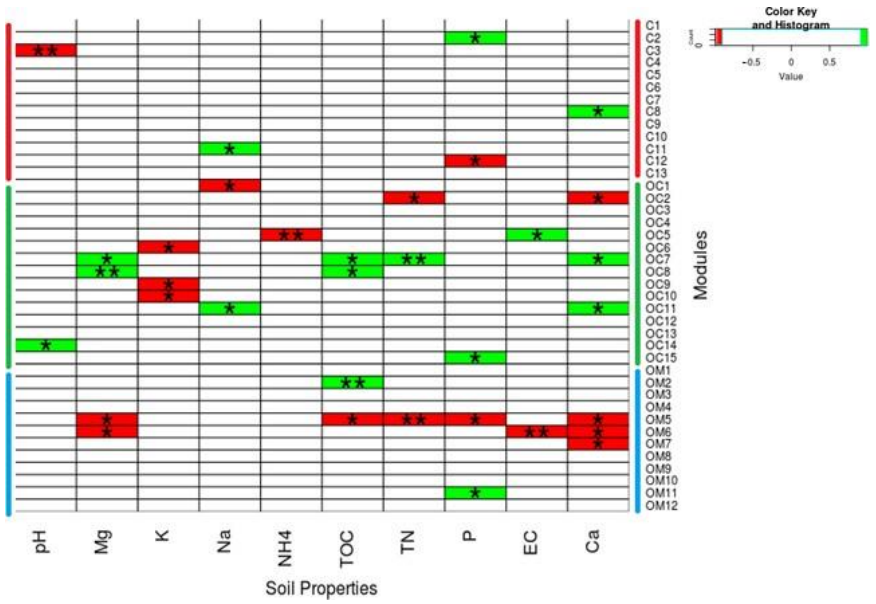

Figure 4. Heat map showing significant module correlation between soil properties and the modules in the Org_C, Org_M and Conv cropping systems. Colors represent positive (green) or negative (red) correlations, and the star symbol (*) in the cells represents the significance of that correlation,with *, $p < 0.05$; **, $p < 0.01$. Conv, conventional cropping system; Org_C, organic cropping system with compost and compost tea; Org_M, organic cropping system with manure; TN, total nitrogen; TOC, total organic carbon; Mg, K, P, Na and Ca; available Mg, K, P, Na and Ca, respectively; EC, electrical conductivity.

4. Discussion

Despite the importance of organic farming in sustainable agriculture, little research has explored the co-occurrence patterns among microbial groups or their functions. In this study, two long-term organic farming systems and a conventional system were studied to explore the co-occurrence patterns among soil bacterial taxa, N cycle functions and their relationship with chemical soil properties, with the aim of deepening our understanding of these microbial communities. Previously, Cuartero et al. [29] studied the microbial species, abundance and diversity of these agricultural systems, observing that the bacterial community structure changed according to the cropping system, although no significant differences were observed in diversity indices.

A microbial network study mainly analyzes the interactions among different microbial species in the soil to maintain ecosystem stability [12], considering that microorganisms are distributed into trophic levels or niches based on their nutritional preferences and functionality [39,40]. Network parameters indicate high modularity values, which means that the microbial community shows modular behavior [41] and can fully adapt to environmental changes [42]. These parameters also indicate habitat heterogeneity, non-random interaction patterns and ecological network complexity [33]. Overall, this approach allowed us to conclude that soil microorganisms tended to co-occur more than would be expected by chance [24]. The bacterial community in the Org_C network is potentially more complex and interconnected than the other two network systems (Org_M and Conv), probably due to the incorporation of compost, which is stable organic matter [43–46]. This sug- gests higher microbial cooperation and a greater exchange of metabolites and information among microbial species, probably due to the creation of favorable and stable niches that

select specific microbial taxa and build up more intensive interactions within the microbial community, which makes those networks more efficient when faced with disturbances. Moreover, a stable, nutrient-rich soil, such as Org_C [29], may contribute to more effective plant growth, nutrient cycling, C utilization, pathogen suppression [47] and the promotion or generation of more functional traits, rather than functional diversification [48]. On the other hand, the decrease in modularity in Conv and Org_M suggests inhibition of microbial functional diversity [49], which could be due to the higher amount of total pesticides in Conv, as previously observed by [50], and the higher pH in Org_M due to the incorporation of fresh organic matter [10,51].

Global networking showed that the cropping system with organic matter had more negative interactions among nodes. Negative interactions could suggest competition, exclusion or even preference for different niches [52,53]. However, according to Coyte et al. [54], negative interactions lead to network stabilization since they compensate for the overexpression of some members, which could lead to destabilization of the network. On the other hand, the eight most abundant OTUs, which showed inverse connections within the global network, also had a greater number of positive connections than within the conventional cropping system. A greater number of positive connections implies cooperativity or codependence among the nodes [54]; more concretely, positive correlations among species mean that these species' coexistence is based on more than chance (e.g., mutualism, predation, etc.). Therefore, our network analysis revealed that an organic-system microorganism habitat with more specialized niches gives stability to the microbial communityand resistance to external factors, with cooperation amongst the most-abundant OTUs promoting the development of the other microorganisms. However, the Conv system showed a higher number of positive connections on its global network, which could indicate a lack of negative regulators, thus being prone to imbalance through external factors.

Modules of highly interconnected nodes [12,55] showed correlations with soil parameters. Org_C showed the highest correlations, indicating the influence of different soil parameters in this network topology, interactions and the potential ecosystem-level functions of the soil. Moreover, the high number of correlations could be due to a greater number of niches in Org_C, which could allow a greater number of different habitats. However, this is not entirely in accordance with the increased network complexity of Org_C, which suggests that module members are less affected by environmental perturba- tions [56,57]. Total nitrogen correlated positively with a module from Org_C (OC7), and this module was composed of Nitrospirae, where members of *Nitrospira* show the capability to perform complete nitrification [58,59], or Actinobacteria, where genera like *Streptomyces* have been linked to some nitrogen-fixation genes [60,61]. The TOC and TN content can be considered drivers of changes in the microbial community in Org_C and Org_M due to the incorporation of organic matter [62,63]. Quilty et al. [64] and Zhou et al. [57] suggested that the amendments had a selective effect on the bacterial community and were more obvious in bacteria than fungi because bacteria are more sensitive to the cropping system and the increase in organic carbon [65].

The identification of keystone bacterial populations is a critical issue in ecology, but is very difficult to achieve due to extreme complexity, high diversity and an uncultivated status [12]. Generalists are the key microorganisms in the microbial network and play important roles in it as predicted from network theory [23]. Generalist habitats have much higher environmental tolerances than specialist habitats, which are more restrictive [66], although generalists normally occupy a small fraction of modules [13,45], as was observed in our study. In our study, the Org_C network had the highest number of generalists, and it would therefore seem logical that this system would show better node communi- cation within and/or among modules than the other two systems. Most generalists were composed of a diverse range of phylogenetic groups typical in soils, such as Proteobacteria, Actinobacteria, Planctomycetes, Chloroflexi, Firmicutes and Verrucomicrobia [67]. However, compared to the conventional cropping system, the incorporation of organic matter into the soil promoted some specific generalists, including Firmicutes (*Bacillus*) and

Verrucomicrobia in Org_M, and Planctomycetes, Chloroflexi and NKB19 in Org_C. Some of these generalists are plant-beneficial microorganisms, such as *Bacillus*, which has been described as a soil-borne pathogen inhibitor [68,69] through the production of antifungal compounds [70], or Planctomycetes and Chloroflexi, which can participate in complex organic matter degradation [71,72], increasing nutrient uptake in plants.

Furthermore, the roles of some nodes shifted in the three networks systems: nodes belonging to Proteobacteria and Actinobacteria, which were considered generalist in the Conv network, were considered specialist in the Org_C and Org_M networks. Generalist and specialist habitats show non-random co-occurrence patterns; furthermore, specialists have a greater and more robust structure than generalists, and these changes could be driven by deterministic processes [73]. This suggests that organic amendments may change the ecological roles of key bacteria [74] due to their wide capacity for adaptation and functionality [75,76].

Our results suggest that changes in bacterial habitats induced by organic matter could lead to different predicted functional groups depending on the new niches generated [77]. Org_C exhibited a higher abundance of different functional groups, which could indicate a more sustainable soil microbial community structure and higher microbial functional-ity [10]. In addition, Org_C showed a large increase in carbon fixation in the prokaryotes pathway, which hosts many kinds of autotrophic bacteria [78], including bacteria with CO_2-fixation capacity. This increase suggests that compost addition could contribute to CO_2 sequestration and storage as described by Ryals et al. [79]. Besides that, the nitrogen flow is considered very efficient and effective when microorganisms are actively transforming the organic nitrogen at the same time that plants are rapidly taking up the NH_4^+ and NO_3^-, as the potential for nitrogen loss is relatively low. In our experiment, N cycling was affected by the different cropping systems, and the abundance of gene families involved could predict the activity of N in the organic and conventional cropping systems, which is crucial, con- sidering nitrogen is a key driver of soil microbial community composition [80]. The higher amount of *nifH*, the key marker gene for nitrogen fixation, in Org_C than in the other sys- tems helps determine soil fertility [81] and could suggest the role of the compost. Moreover, fresh organic matter and inorganic fertilizer would inhibit the predicted N-fixation genes of specific N-fixer groups [82,83]. Genes involved in denitrification were more abundant than nitrification genes. However, genes involved in the nitrification process—principally hydroxylamine oxidoreductase (*hao*) involved in conversion of (NH_2OH) in nitrous oxide (N_2O)—were more abundant in Org_C. Denitrification is the basic avenue for nitrogen loss in agricultural soils [84,85], and the genes *narG*, *nirK*, *norB*, *nosZ* and *nrf* are involved in the conversion of nitrite to nitrogen gas. Org_C and Org_M showed lower values than Conv, although *nosZ*, the enzyme known to catalyze the last step of denitrification, the conversion of nitrous oxide (N_2O) to nitrogen gas (N_2), was higher in Org_C. This could indicate a decrease in N_2O emissions [86]. Moreover, Org_C showed a higher predicted abundance of gene *nrfA*, related to nitrate reduction to ammonium (DNRA), which is beneficial to N retention and immobilization in agricultural soils since N is converted to NH_4^+ rather than lost through denitrification and anammox [87].

5. Conclusions

In this work, we provide insight into a soil bacterial community affected by differ- ent long-term cropping systems via network interaction analysis and functional analysis, principally N cycling. The network revealed how long-term compost application modified the bacterial community, increasing the network complexity and enhancing modulation and communication through generalists to a greater extent than in the Conv and Org_M cropping systems. In addition, changes in these bacterial habitats could have also altered bacterial functions, since Org_C showed higher predicted nitrogen-fixing potential, de- creased N_2O emissions and greater carbon-sequestration potential than the other cropping systems. This implies the importance of using a stable organic amendment as compost and

the use of adequate tools to study the response of the bacterial community to long-term cropping systems.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12020316/s1, Table S1: Management characteristics of the three cropping systems; Table S2: Abundance of phyla in the different modules (expressed in percent); Table S3: Subnetworks of the eight most abundant OTUs and their first neighbor nodes in the three cropping systems; Table S4: Classification of generalists in the three cropping systems; Table S5: Predicted functions of the bacterial communities found in the three cropping systems (relative abundances); Table S6: Mantel analysis of relationships between bacterial community and soil properties and N cycling genes.

Author Contributions: Conceptualization, J.C., V.S.-N., R.Z., J.W., J.A.P., J.-M.V. and M.R.; methodology, J.C., O.Ö., V.S.-N., J.W., R.Z., J.A.P., J.-M.V. and M.R.; validation, R.Z., J.A.P. and M.R.; formal analysis, J.C. and J.-M.V.; investigation, J.C., O.Ö., V.S.-N., J.W., R.Z., J.A.P., J.-M.V. and M.R.; resources, J.C., O.Ö., V.S.-N., J.W., R.Z., J.A.P., J.-M.V. and M.R.; data curation, J.C. and J.-M.V.; writing—original draft preparation, J.C.; writing—review and editing, J.A.P., J.-M.V. and M.R.; visualization, J.C. and J.-M.V.; supervision, J.A.P., J.-M.V. and M.R.; project administration, J.A.P. and M.R.; funding acquisition, J.A.P. and M.R. All authors have read and agreed to the published version of the manuscript.

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Supplementary Material of Long-term compost amendment changes interactions and specialization in the soil bacterial community, activating beneficial N-cycling genes in the soil.

Long-term compost amendment changes interactions and specialization in the soil bacterial community, activating beneficial N-cycling genes in the soil.

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Table S1. Management characteristics of the three cropping systems.

Cropping 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)	*Brassica oleracea var. sabellica*		
Harvest	Manual on 20-25 February 2018. Crop residues were incorporated into the soil.		
Crops (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; 15,000 kg ha^{-1} sheep compost tea*	
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.

Table S2. Abundance of phyla in the different modules (expressed in percent)

Phylum	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13
Acidobacteria	7.7	1.25			4.35		22.22	5.88				9.09	3.89
Actinobacteria	17.95	20	38.46	35	30.43	30	11.11	11.76	41.67	42.85	29.41	9.09	15.58
Bacteriodetes	7.69	6.25	7.69	10	8.70	5	11.11	11.76				18.18	24.67
BRC1	2.56												
Chloroflexi	7.69	1.25	3.85					11.76			11.76		3.89
Firmicutes	10.26	16.25				5	11.11				35.29	9.09	3.89
Proteobacteria	43.59	37.5	30.77	25	56.52	50	22.22	41.18	41.67	28.57	35.29	45.45	33.77
TM7	2.56	2.5								14.29			
Cyanobacteria		1.25								14.28			
Elusimicrobia		1.25	3.85										
Gemmatimonadetes		7.5						5.88					1.30
Nitrospirae		1.25											1.30
Plantcomycetes		2.5		15			11.11	5.88	8.33				
Verrucomicrobia		1.25	3.85			5			8.33		5.88		7.79
Armatimonadetes			3.85										1.29
NKB19			3.85										1.30
OP11			3.85										
Kazan-3B-28				5									
FBP						5							
Chlorobi							11.11						1.30
WS3								5.88					
Thermi											5.88		
TM6												9.09	
Total nodes	39	80	26	20	24	20	9	17	12	7	17	11	77

Phylum	OC1	OC2	OC3	OC4	OC5	OC6	OC7	OC8	OC9	OC10	OC11	OC12	OC13	OC14	OC15
Acidobacteria	2.13		4.25	10.53		3.7			6.25	25		33.33		14.28	
Actinobacteria	27.66	36.11	14.89	10.53	25	33.30	33.33	27.27	8.75	25	16.67	16.67	30	14.28	50
Bacteriodetes	6.38	16.67	6.38	15.79		3.70			5				20	7.14	
BRC1							11.11								
Chloroflexi	10.64					7.41			2.5				10		
Firmicutes	6.38	11.11	8.51	5.26	12.5	14.81		9.09	7.5						
Proteobacteria	40.42	25	42.55	36.84	56.25	22.22	33.33	54.54	35	25	83.33	33.33	30	64.29	25
TM7	2.12	2.78	2.13												
Cyanobacteria				5.26		3.70			1.25						12.5
Elusimicrobia									3.75						
Gemmatimonadetes		2.78	2.13				11.11	9.09	8.75	12.5					
Nitrospirae							11.11		1.25						
Planctomycetes		2.78	8.51		6.25				8.75				10		12.5
Verrucomicrobia	2.13	2.78	2.13	10.53					6.25	12.5					
Armatimonadetes			2.13						3.75						
NKB19			2.13												
Chlorobi			4.25			3.70									
Thermi	2.13														

BHI80-139				5.26											
OD1						3.70									
GN02						3.70									
OP3									1.25						
Total nodes	47	36	47	19	16	27	9	11	80	8	6	6	10	14	8

Phylum	**OM1**	**OM2**	**OM3**	**OM4**	**OM5**	**OM6**	**OM7**	**OM8**	**OM9**	**OM10**	**OM11**	**OM12**
Acidobacteria			3.45	11.54		8.51			3.70	28.57	10.34	6.67
Actinobacteria	36.36	13.79	10.34	7.69	42.10	23.40	25	35.29	11.11		13.79	13.33
Bacteriodetes	6.06		27.59			6.39	9.38	5.88	3.70		6.90	6.67
BRC1						2.13						
Chloroflexi		6.90	3.45			2.13	12.5		11.11	14.29	3.45	
Firmicutes		20.69			5.26	4.25	12.5		7.41	14.29	3.45	13.33
Proteobacteria	45.45	44.83	41.38	38.46	36.84	27.66	34.38	17.65	48.15	14.29	37.93	46.67
TM7			3.45					5.88				
Cyanobacteria	3.03					4.25		5.88				
Elusimicrobia	3.03		3.45			2.13						
Gemmatimonadetes	3.03			19.23		6.38	3.13				6.90	6.67
Nitrospirae				3.85				5.88				
Planctomycetes	3.03	10.34	3.45	7.69	10.53	4.25		5.88	3.70		3.45	
Verrucomicrobia		3.45	3.45	3.84		2.13	3.13	5.88	3.70		10.34	
Armatimonadetes						2.12		5.88				
NKB19								5.88				
Chlorobi					5.26					14.29		
Thermi									3.70			
OP11									3.70			
WS3						2.13						
WPS-2										14.29		
Fibrobacteres				3.85								
OD1											3.45	
Tenericutes												6.67
OP3						2.13						
Total nodes	33	29	29	26	19	47	32	17	27	7	29	15

(C) Conv, Conventional system; (OC) Org_C, Organic cultivation with sheep manure compost and compost tea; (OM) Org_M, Organic cultivation with sheep manure.

Table S3. Subnetworks of the eight most abundant OTUs and their first neighbor nodes in the three cropping systems

	Cropping System Nodes			Cropping System Edges			
OTU	**Conv**	**Org_C**	**Org_M**	**Conv**	**Org_C**	**Org_M**	**Phyla**
436	3	7	4	2 np – 1 pp	1 np – 6 pp	1 np – 3 pp	Proteobacteria
605	7	-	-	1 np – 6 pp	-	-	Proteobacteria
110	2	9	4	1 np – 1 pp	5 np – 4 pp	1 np – 3 pp	Actinobacteria
003	1	3	13	1 pp	1 np – 2 pp	13 pp	Acidobacteria
324	8	1	6	8 np	1 pp	6 pp	Gemmatimonadetes
438	1	6	4	1 np	2 np – 4 pp	3 pp	Proteobacteria
486	8	8	4	6 np – 2 pp	4 np – 4 pp	1 np – 3 pp	Proteobacteria
174	5	-	3	3 np – 2 pp	-	3 pp	Bacteriodetes
426	-	12	-	-	4 np – 8 pp	-	Proteobacteria
172	-	-	3	-	-	3 pp	Bacteriodetes

np, negative edges; pp, positive edges; - none detected; Conv, Conventional system; Org_C, Organic cultivation with sheep manure compost and compost tea; Org_M, Organic cultivation with sheep manure

Table S4. Classification of generalists in the three cropping systems

Cropping system	OTU ID	Generalist	Phylum	Class	Order	Family	Genus
Conv	OTU108◎	Module Hub	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	*Nocardioides*
Conv	OTU153◎◎	Module Hub	Actinobacteria	Rubrobacteria	Rubrobacterales	Rubrobacteraceae	*Rubrobacter*
Conv	OTU422◎◎	Module Hub	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Unclassified	*Unclassified*
Conv	OTU483◎◎	Module Hub	Proteobacteria	Betaproteobacteria	Methylophilales	Methylophilaceae	*Methylotenera*
Conv	OTU085◎◎	Connector	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Unclassified
Conv	OTU442	Connector	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingopyxis*
Org_C	OTU065◎*	Module Hub	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	*Agromyces*
Org_C	OTU161◎	Module Hub	Actinobacteria	Thermoleophilia	Solirubrobacterales	Solirubrobacteraceae	Unclassified
Org_C	OTU239	Module Hub	Chloroflexi	Chloroflexi	Herpetosiphonales	Unclassified	Unclassified
Org_C	OTU339*	Module Hub	NKB19	Unclassified	Unclassified	Unclassified	Unclassified
Org_C	OTU345*	Module Hub	Planctomycetes	BD7-11	Unclassified	Unclassified	Unclassified
Org_C	OTU599◎*	Module Hub	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Arenimonas*
Org_C	OTU461	Connector	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	*Ramlibacter*
Org_C	OTU508	Connector	Proteobacteria	Deltaproteobacteria	Desulfuromonadales	Geobacteraceae	*Geobacter*
Org_M	OTU024◎	Module Hub	Actinobacteria	Acidimicrobiia	Acidimicrobiales	AKIW874	*Unclassified*
Org_M	OTU271◎	Module Hub	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*
Org_M	OTU485	Module Hub	Proteobacteria	Betaproteobacteria	MKC10	Unclassified	Unclassified
Org_M	OTU512◎	Module Hub	Proteobacteria	Deltaproteobacteria	Unclassified	Unclassified	Unclassified
Org_M	OTU537*	Module Hub	Proteobacteria	Deltaproteobacteria	Spirobacillales	Unclassified	Unclassified
Org_M	OTU630◎*	Module Hub	Verrucomicrobia	Pedosphaerae	Pedosphaerales	OPB35	Unclassified

Conv, Conventional cropping system; Org_C, Organic cropping system with compost and compost tea, Org_M, Organic cropping system with manure. "◎" indicates that the node was shared by Org_C such as specialist; "◎" indicates that the node was shared by Org_M such as specialist; and "*" indicates that the node was shared by Conv such as specialist.

Table S5. Predicted functions of the bacterial communities found in the three cropping systems (relative abundances)

Metabolic pathway	Conv	Org_C	Org_M	Anova	Kruskal-Wallis
Amino acid metabolism	0.23±0.00 ab	0.24±0.00 a	0.19±0.01 b	-	**
Amino acid related enzymes	1.23±0.04 a	1.22±0.03 a	1.08±0.07 b***	**	-
Bacterial toxins	0.06±0.00 ab	0.07±0.01 a	0.04±0.01 b	-	**
Biosynthesis and biodegradation of secondary metabolites	0.07±0.00 ab	0.08±0.01 a	0.06±0.01 b	-	**
Carbon fixation photosynthetic organism	0.41±0.02 ab	0.44±0.01 a	0.34±0.03 b	-	**
Carbon fixation in prokaryotes	0.98±0.03 b	1.18±0.03 a***	0.62±0.01 c***	***	-
Nitrogen metabolism	0.69±0.03 a	0.73±0.02 a	0.50±0.04 b***	***	-
Lipid metabolism	0.10±0.00 ab	0.12±0.01 a	0.08±0.00 b	-	**
Nucleotide metabolism	0.03±0.00 a	0.03±0.00 a	0.02±0.00 b	-	**
Penicillin and cephalosporin biosynthesis	0.07±0.00 a	0.07±0.00 a	0.05±0.00 b	-	**
Phosphotransferase system PTS	0.08±0.00 ab	0.09±0.00 a	0.06±0.00 b	-	**
Signal transduction mechanism	0.42±0.02 ab	0.45±0.02 a	0.32±0.01 b	-	**
Tetracycline biosynthesis	0.13±0.01 ab	0.13±0.00 a	0.12±0.00 b	-	**
Toluene degradation	0.20±0.01 ab	0.23±0.01 a	0.13±0.01 b	-	**
Sulfur metabolism	0.34±0.01 ab	0.37±0.02 a	0.26±0.01 b		**
Benzoate degradation	0.55±0.03 b	0.70±0.02 a***	0.59±0.03 b	***	-
DNA replication proteins	0.76±0.01 b	0.80±0.01 a***	0.48±0.01 c***	***	-
Phenylalanine, tyrosine and tryptophan biosynthesis	0.58±0.01 ab	0.71±0.01 a	0.52±0.00 b	-	**

(mean±sd; n=5 values are expressed with an e-value of 1x10-2). The mean value followed asterisks in each cropping system (*, **, ***) represents significant differences with respect to the conventional cropping system (Conv) by Dunnett's test (*P < 0.05; **P < 0.01, ***P < 0.001, respectively). The mean value followed by different letters represent significant differences between systems by Tukey's test or Dunn's Kruskal-Wallis Multiple Comparison test. Conv, Conventional cropping system; Org_C, Organic cropping system with compost and compost tea; Org_M, Organic cropping system with manure.

Table S6. Mantel analysis of the relationships between the overall bacterial community and soil properties and N cycling genes.

	Network Topology					
	Conv		Org_C		Org_M	
	r_M	P	r_M	P	r_M	P
Soil properties	0.09133	**	0.06448	**	0.07088	**
Functionality (N cycling genes)	0.2121	**	-0.001593	0.518	-0.01519	0.728

Significant P values are indicated as *P < 0.05; **P < 0.01, ***P < 0.001, respectively. Conv, Conventional cropping system; Org_C, Organic cropping system with compost and compost tea; Org_M, Organic cropping system with manure; r_M Mantel statistic r.

A first-year melon/cowpea intercropping system improves soil nutrients and changes the soil community

Abstract

Intercropping is a practice involving the simultaneous growing of two or more crops at the same time on the same soil. Different types of intercropping and combined systems have been proposed, but not all constitute an improvement on yield and soil quality since there must be a balance on the combined crops. In Mediterranean area as Murcia, intercropping of melon (*Cucumis melo L.*) highly cultivated in the region of Murcia, and cowpea (*Vigna unguiculata L. Walp*) a crop that increase nitrogen uptake by nodulation, and adapted to low fertility requirements, can contribute significantly to overcome the challenges of developing both productive and environmentally friendly agricultural system. In this sense, microorganisms play an important role such a nutrient cycle, organic matter turnover or soil-borne pathogen suppression.

To study the effects of melon/cowpea on soil properties and bacterial community, five different cropping system are assayed for a year, consisted on three types of intercropping systems attending to the disposal of both crops: a) mixed melon cowpea (MC1); b) row melon:cowpea 1:1 (MC2); and c) row melon:cowpea 2:1 (MC3); in comparison to the

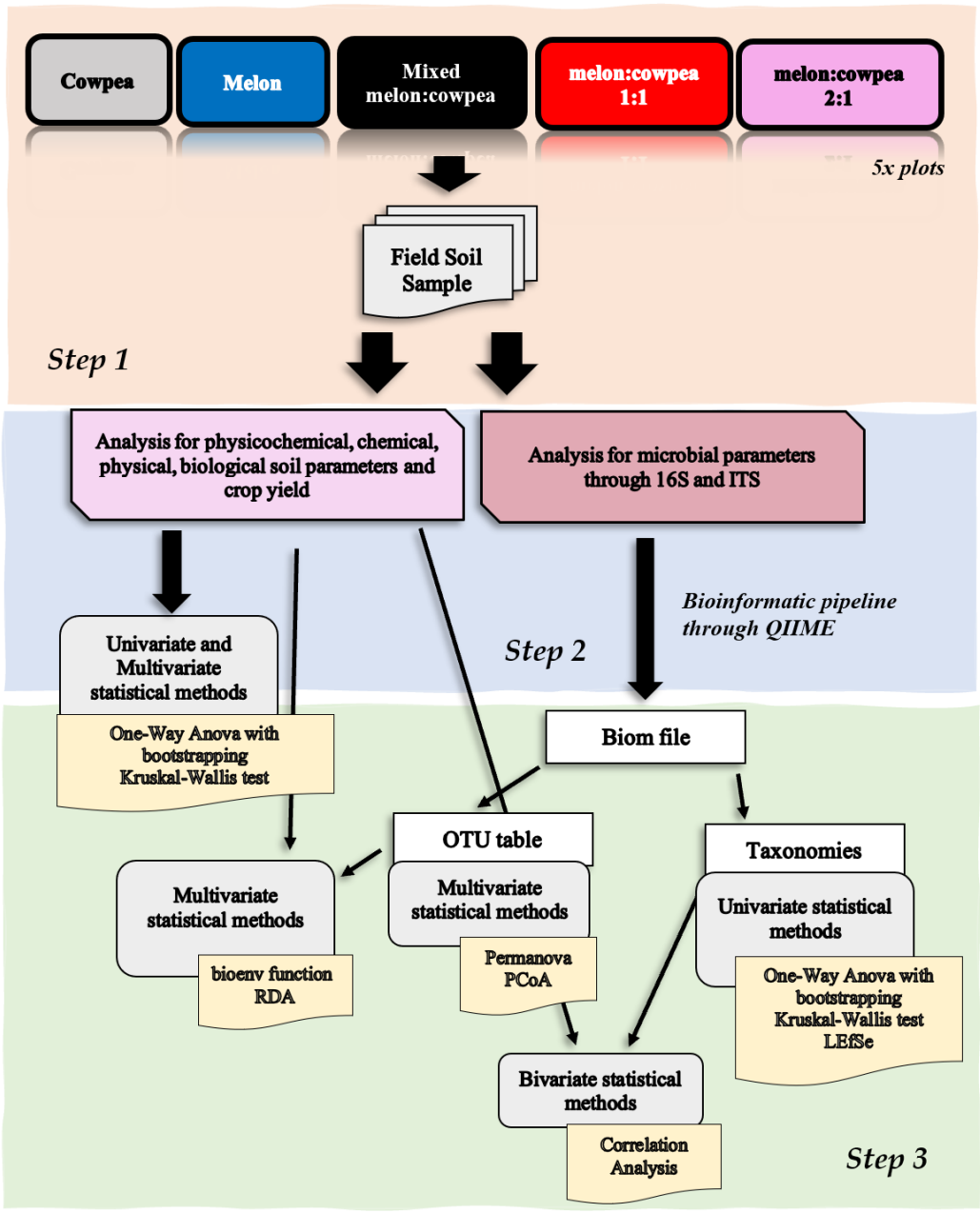
both monocrop systems d) melon monocrop (M); and e) cowpea monocrop (C). The amount of fertilizer is reduced by 30% in the intercropping systems compared with the melon monocrop that are maintained as commercial fertilization rate. Some different physicochemical and chemical parameters, enzyme activities and some molecular parameters such as gene expression related to the N cycle (*amoA*, *narG* and *nirK*) and bacterial composition and diversity through NGS technologies using 16S rRNA genes have been measured.

To test the differences between soil properties, enzymatic activities and gene expression, analysis of Variance (ANOVA) evaluating the stability of their results via bootstrapping (using 100 replicates) followed by Tukey's honestly significant difference and Dunnett's comparisons for the control system (using M such a control) or Kruskal-Wallis test followed by Dunn test when normality is not met, are used. From amplicon data, α- and β-diversities are calculated, and the differences are evaluated through ANOVA or Kruskal-Wallis test for α-diversity whereas a Permutational Multivariate Analysis of Variance (PERMANOVA) with 999 permutations is performed to study the differences on the β-diversity which is represented through Principal Coordinates Analysis (PCoA). Each taxon at phylum and genus level is tested in the same way than soil properties. Linear Discriminant Analysis effect size (LEfSe) algorithm is performed to get the possible biomarkers from soil under different patterns in an intercropping system.

The results show that in just only one year of intercropping increase melon yield, and soil properties are highly affected through increasing TN, NH_4^+, TOC and available P compared to melon monocrop M. Regarding to microbial activity, the results reveal that intercropping system has the higher β-glusosidase activity compared with melon monocrop (M). β-diversity represents by PCoA evidenced differences between intercropped and monocrops, which is confirmed by PERMANOVA. Redundancy Analysis (RDA) reveal on intercropped systems a relationship among bacteria community structure, TN, P content and melon yield. *Pseudomonas* is significantly higher in the intercropped systems than in the monocrop (M and C), *Thauera* in MC1 or *Sphingomonas* and *Skermanella* in MC2. A LEfSe analysis shows different bacteria as key microorganisms for each of the intercropped systems. In general, the log copies of these three genes (*amoA*, *narG* and *nirK*) are lower in intercropped systems compared to monocropping systems (M and C).

Relevant contributions reach with this paper are that after just a year of intercropping system, soil properties and bacterial regardless of cowpea distribution are improved. Through bioinformatic methods, it is possible to reveal the above differences, indicating an increase in nutrients and some microbial activities. In addition, it is possible to find a relationship between the yield and soil microbial community through multivariate techniques. Nevertheless, although the computational statistical techniques employed allow to detect where the changes occurred, further study would be necessary to see the influence on soil of cowpea distribution, as well if a long-term melon:cowpea intercropping could maintain these benefits to soil.

Workflow


Cowpea
Melon
Mixed melon:cowpea
melon:cowpea 1:1
melon:cowpea 2:1
5x plots
Field Soil Sample
Step 1
Analysis for physicochemical, chemical, physical, biological soil parameters and crop yield
Analysis for microbial parameters through 16S and ITS
Bioinformatic pipeline through QIIME
Step 2
Univariate and Multivariate statistical methods
One-Way Anova with bootstrapping Kruskal-Wallis test
Biom file
OTU table
Taxonomies
Multivariate statistical methods
bioenv function RDA
Multivariate statistical methods
Permanova PCoA
Univariate statistical methods
One-Way Anova with bootstrapping Kruskal-Wallis test LEfSe
Bivariate statistical methods
Correlation Analysis
Step 3

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A first-year melon/cowpea intercropping system improves soil nutrients and changes the soil microbial community

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ABSTRACT

The melon/cowpea intercropping system can be a specific and efficient cropping pattern in a horticultural field. Intercropping systems contribute to the optimization of land use, fostering sustainable and efficient agriculture. This study entails a first-year comparative intercropping assay using cowpea (*Vigna unguiculata*) and melon (*Cucumis melo*) under organic management with different patterns and 30% less organic fertilization than usual in monocrops. We determined the soil nutrients, physicochemical properties, enzyme activities and microbes by high-throughput sequencing. We found that the intercropping system changed the bacterial community structure independently of the intercropping pattern. The bacterial community was characterized by a higher abundance of the phyla Proteobacteria and Bacteroidetes phyla and of the genus *Pseudomonas*, which are related to nutrient cycling, and by greater amounts of other beneficial microorganisms like *Bacillus, Streptomyces* and *Sphingomonas*. The intercropped systems significantly boosted the total nitrogen, available phosphorus and total organic carbon levels in addition to the melon yield. They also enhanced the acid phosphatase and β-glucosidase activity compared to the melon monocrop. Results from this study suggest that melon/cowpea intercropping, starting from the first year, not only provides a stable supply of food and income due to the diversified cropping systems, but is also beneficial for the soil microbial community and environment.

1. Introduction

Intercropping is a practice involving the simultaneous growing of two or more crops on the same land during the same growing season (Zhou et al., 2011). This practice is becoming increasingly important for maintaining and increasing soil quality and subsequently crop productivity (Singh et al., 2016). Intercropping has demonstrated advantages, including efficient nutrient acquisition; reduced pest, disease and weed damage; improved microbial diversity; and improved utilization of land resources (Mousavi and Eskandari, 2011). Different types of intercropping and combined systems have been proposed, but not all intercropping systems constitute improvements, since there must be a balance among the crops used (Gebru, 2015). It is particularly important to not use crops that compete for physical space, nutrients, water, or sunlight, and the environmental conditions in a given area and the crops or va- rieties available must also be taken into account (Lithourgidis et al., 2011). Maize is one of the predominant intercrops used, often combined with legume crops (Manasa et al., 2018). This combination makes it possible to develop an energy-efficient and sustainable system, as the legumes have an N-fixing capability and more protein-yielding potential in the form of either grain or forage (Maitra et al., 2019). In arid envi- ronments, the legume crop cowpea (*Vigna unguiculata L. Walp*) is nor- mally used because of its adaptability and low fertility requirements,and it can improve legume nitrogen uptake by nodulation (Li et al.,

Abbreviations: M, Melon monocrop; MC1, Mixed intercropping, with melon mixed with cowpea in the same row; MC2, Row intercropping at a ratio of 1:1 (melon: cowpea), alternating one melon row and one cowpea row; MC3, Row intercropping at a ratio of 2:1 (melon:cowpea), alternating two melon rows and one cowpea row; C, Cowpea monocrop.

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2007). Therefore, it can be intercropped not only with maize, but also millet, sorghum, and some other crops (Chimonyo et al., 2016; Nelson et al., 2018).

Melon (*Cucumis melo* L.) is the main export crop in the region ofMurcia (57%). Intensive melon cultivation can generate soil and water degradation due to the excessive use of pesticides to reduce the impact of pathogens and the necessary application of synthetic fertilizers due to nutrient depletion (Li, 2001). Intercropping melon and cowpea could contribute significantly to overcoming the challenges of developing both productive and environmentally friendly agricultural systems for melon cultivation. In addition, previous studies have reported that the planting pattern could also affect the soil and yield (Raza et al., 2019; Xianhaiet al., 2012), so it is necessary to study intercropping as well as plant distribution.

The interactions among microbes, nutrients and enzymes in intercropping systems lead to an increase or decrease in microbe quantity and enzyme activity, contributing to the improvement of the soil microecological environment (Zhou et al., 2019). Soil microorganisms are key drivers of many soil biological, chemical, and physical processes, such as soil structure formation, the nutrient cycle, organic matter turnover, toxin accumulation or removal, and soil-borne pathogen suppression (Bever et al., 2012; Blagodatskaya and Kuzyakov, 2013). Several studies have investigated the changes in the microbial characteristics of soils caused by intercropping (Jin et al., 2020; Li and Wu, 2018). However, changes in the soil microbial community resulting from melon-cowpea intercropping have not been studied in depth. We hypothesized that intercropping would improve crop yield, increase soil bacterial diversity and enzyme activities and change the soil community structure. In this paper, our objective was to investigate physico-chemical properties, nutrient content, enzyme activities and the bacterial community resulting from three different types of melon-cowpea intercropping systems in their first year. We also wished to determine the relationship between these changes and soil chemical properties and crop yield compared to monoculture systems.

2. Materials and methods

2.1. Experimental design and sampling

An intercropping experiment with melon and cowpea was performed under organic conditions in La Palma (Cartagena) (37° 41 18 N 0° 56 60 W), a province of Murcia (S.E. Spain), in May–August 2018. The field trial was conducted in a soil that had been uncultivated for at least the last five years prior to the study; the soil was classified as Haplic Calcisol (Loamic, hypercalcic) (WRB, I.U. of S.S.W.G, 2015). The climate in the area of study is semiarid Mediterranean, with a mean annual tempera- ture of 18 °C, a mean annual precipitation of 275 mm and an annual potential evapotranspiration of 900 mm.

The assayed treatments were as follows: (*i*) melon (*Cucumis melo)* monocrop (M); (*ii*) cowpea (*Vigna unguiculata)* monocrop (C); (*iii*) mixed intercropping, with melon mixed with cowpea in the same row (MC1); (*iv*) row intercropping at a ratio of 1:1 (melon:cowpea), alternating one melon row and one cowpea row (MC2); and *(v*) row intercropping at a ratio of 2:1 (melon:cowpea), alternating two melon rows and one cowpea row (MC3). The field experiment was a completely randomized design with three plots per treatment, and each plot had a surface area of 120 m^2. Melon seedlings were planted at a density of 0.4 plants per m^{-2}, with a spacing of 200 cm between rows and 120 cm between plants in both the monocropped and intercropped systems. The density of cowpea plants was 2.5 plants per m^{-2} and 1.5 plants per m^{-2} in the 1:1 row (MC2) and 2:1 row (MC3) systems, respectively. In the intercropped row systems, the cowpea rows were spaced 100 cm from the melon rows, and there were 20 cm between cowpea plants in the same row. In the mixed system (MC1), the cowpea density was 0.4 plants per m^{-2} with one cowpea plant between melon plants in each row and spacing of 200 cm between rows and 120 cm between plants. The melon density was thus the same in the different treatments, but the cowpea density changed(Fig. 1).

All crops were drip irrigated and grown under organic management. The melon plot (M) received the equivalent of 3000 kg ha^{-1} of organic fertilizer (N org) (3.2% N and 7% K_2O), and the cowpea plot (C) received the equivalent of 1875 kg ha^{-1} of Norg. The intercropped plots (MC1, MC2 and MC3) received 30% less Norg than the melon monocrop to assess the efficiency of the intercropping in reducing external fertiliza- tion needs. The melons and cowpeas were simultaneously harvested twice, on July 31, 2018 and August 6, 2018. The harvest was carried out manually, as is the tradition in the area, to avoid damaging the melon fruits.

Five random soil subsamples (0–10 cm depth) were collected with an auger from the plots on August 10, 2018, just after harvest. Soil samplesin MC2 and MC3 were only collected from the melon rows. The samples were taken between two adjacent plants in all cases. The soil samples were separated into two aliquots, one of which was kept at ambient temperature for chemical analyses and the other stored in a cool boxwith ice for biological analysis. All samples were taken to the lab immediately. The soil was air-dried for one week for chemical analyses and sieved at < 2 mm. Soil for biological analysis was sieved at < 2 mm once in the lab and stored at — 20 °C.

2.2. Soil properties and enzyme activities

The soil pH and electrical conductivity (EC) were measured in deionized water (1:5 w/v). The total organic carbon (TOC) and total nitrogen (TN) were determined using an elemental CHNS-O analyzer (EA-1108, Carlo Erba). Soil NH_4^+ was extracted with 2 M KCl in a 1:10 soil:extractant ratio and measured by colorimetric assay following Kandeler and Gerber (1988) and Keeney and Nelson (1983). Available P (P) was measured using the Olsen method (Olsen, 1954). Available nutrients were measured using ICP-MS (Agilent 7500CE).

Phosphatase and β-glucosidase activities were measured using the a fluorogenic approach according to Marx et al. (2001), and dehydroge- nase activity was measured via a colorimetric procedure according to Von Mersi and Schinner (1991).

2.3. Soil DNA extraction, PCR amplification 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 the 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 via the next-generation sequencing of bacterial 16 S hypervariable regions using an Ion Torrent™ Personal Genome Machine™ (PGM) System. Bacterial 16 S regions were amplified using an Ion 16 S™ Metagenomics Kit (Thermo Fisher Scientific) with two different degenerate primer sets to amplify regions V2–8 and V3–6, V7–9. The amplified 16 S amplicons were then processed using an Ion Xpress™ Plus Fragment Library Kit in combi- nation with an Ion Xpress™ Barcode Adapter 1–96 Kit (Thermo Fisher Scientific). All purification processes between incubation and amplifi- cation reactions of the library preparation were processed using DynaMag™ 2 magnetic racks (Thermo Fisher Scientific) and an AMPure XP Purification Kit (Beckman Coulter). After library prepara-tion and barcoding, we determined the size and concentration of thefinal libraries using an Agilent 2100 Bioanalyzer system and the Agilent High Sensitivity DNA kit. The 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 SequencingKit (Thermo Fisher Scientific).

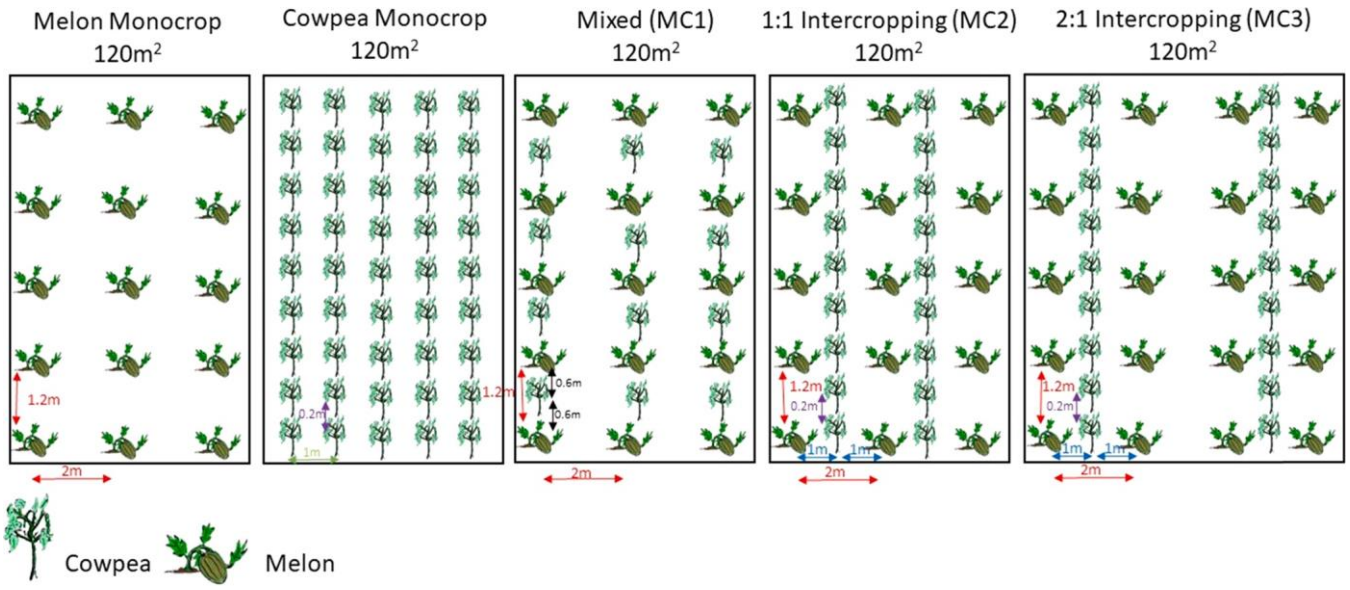

Fig. 1. Planting framework of melon and cowpea intercropping. Distance among rows of melon was 2 m while among melons in line was 1.2 m. In the intercropping (MC2 and MC3), the cowpea plants were arranged between the rows of melon with a separation of one meter between rows. In the MC1, cowpea plants were ar- ranged in the same row, with a separation of 0.6 m among melon plants.

2.4. Sequencing data processing

Bacterial raw sequences, barcodes and primers were trimmed according to the BaseCaller application. The sequences were denoised with ACACIA (Bragg et al., 2012), and low quality sequences were discarded using the Quantitative Insights into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010) from the Microbiome Helper Virtual Box (Comeau et al., 2017). 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 (Rogneset al., 2016) with the ribosomal database project (RDP database). Low-confident OTUs were removed.

The sequences were uploaded to the European Nucleotide Archive (ENA) with the study accession code PRJEB42624.

2.5. Statistical analysis

All tests were performed using R language (Team, 2020). Normality and homogeneity of variance assumptions were assayed by the Shapiro-Wilk and Levene's tests using the car (Fox et al., 2007) package. Mean comparisons were performed with one-way analysis of variance (ANOVA) followed by post-hoc tests, Tukey's honestly significant dif-ference (HSD) for all-pair comparisons and Dunnett's comparisons for the control system. In the cases in which homoscedasticity was not met, Welch's test was performed using the 'pairwise.t.test' function with Bonferroni-Holm corrections for multiple comparisons. The robustness of the estimations was checked by the bootstrapping approach using 100 replicates. When data did not fit a normal distribution, non-parametric Kruskal-Wallis tests were performed, and if the assayed data were sig- nificant, a multiple comparison Z-values test was performed using the 'dunnTest' function with Benjamini-Hochberg corrections in the FSA package (Ogle and Ogle, 2017).

Bacterial alpha diversity [Chao1 as richness and Shannon (H') as diversity index] was estimated on rarefied microbial data using the vegan package (Oksanen et al., 2007).

A linear discriminant analysis (LDA) effect size (LEfSe) pipeline (Segata et al., 2011), available at http://huttenhower.sph.harvard.edu/galaxy/, was used with the default parameters at all taxonomic levels to identify genera that were differentially abundant among the cultivation systems. Three different steps were performed using the following algorithm: (i) a nonparametric Kruskal-Wallis test to detect the statistical differences between abundances; (ii) a pairwise test among subclasses using the Wilcoxon rank-sum test to evaluate biological consistency; and (iii) an LDA to estimate the effect size between abundances.

Principal coordinates analysis (PCoA) was used to visualize the variation in community composition by cultivation system based on the Bray-Curtis distance. To evaluate differences between the cropping systems, a Permutational Multivariate Analysis of Variance (PERMANOVA) was conducted using the 'betadisper' and 'adonis' functions with 999 permutations from the vegan package, followed by the 'pairwise.adonis' function with Benjamini-Hochberg corrections for multiple comparisons between specific cultivation systems from the pairwiseAdonis package (Arbizu, 2017) when the homogeneity of variance assumption was met. In the cases in which homoscedasticity was not fulfilled, an Analysis of Similarities (ANOSIM) was carried out instead. Relationships between the bacterial community and the rest of the parameters were determined using the 'bioenv' function from the vegan package to find the best subset of parameters (using Euclidean distance) that had a maximum correlation with the community dissimilarity matrix (Clarke and Ainsworth, 1993). Redundancy analysis (RDA) was performed through the vegan package to visualize the correlation between OTUs and physico-chemical, biological and harvest parameters. The OTU abundance was Hellinger transformed prior to analysis with the retained variables from the bioenv procedure (Legendre and Gal-lagher, 2001), which was performed via the 'bioenv' function based on Spearman's rank correlation coefficient. To equalize the number of replicates for 'bioenv' and 'rda', the function 'sample_n' in the dplyr package (Wickham et al., 2019) was used.

3. Results

3.1. Effects of intercropping on crop yield

The intercropped melon systems showed a higher melon yield (34% 74%)–than the melon monocrop (M), and the yield was significantly higher in MC1 and MC3. We also observed a greater number of melons in the intercrops (MC3 52%, MC1 40% and MC2 33%) than in the monocrop (M) (Table 1). The cowpea yield, on the other hand, was

Table 1
Soil properties and crop yield in the intercropping systems.

Physico-chemical and chemical soil properties	C	M	MC1	MC2	MC3	Anova	Kruskal-Wallis
pH	8.4 ± 0.0	8.5 ± 0.0	8.4 ± 0.0	8.3 ± 0.0	8.4 ± 0.0	ns	–
EC (µS cm^{-1})	307 ± 6	290 ± 2	332 ± 30	298 ± 17	299 ± 37	–	ns
TOC (g kg^{-1})	11.8 ± 0.3 a	9.5 ± 0.1 b	11.2 ± 0.4 ab	11.1 ± 0.2 ab	11.9 ± 0.2 a	–	*
TN (mg kg^{-1})	1.3 ± 0.0 a	1.1 ± 0.0 b	1.3 ± 0.0 a	1.3 ± 0.0 a	1.3 ± 0.0 a	–	*
NH_4^b (mg kg^{-1})	0.53 ± 0.18 b	0.88 ± 0.00 ab	1.83 ± 0.10 ab	3.36 ± 0.63 ab	4.48 ± 0.72 a	–	* *
Ca (mg kg^{-1})	1579 ± 236 a	1540 ± 39 a	1432 ± 297 a	908 ± 77 b* *	951 ± 22 b* *	* *	–
Mg (mg kg^{-1})	360 ± 75 ab	325 ± 62 ab	426 ± 93 a	244 ± 35 b	242 ± 4 b	*	–
K (mg kg^{-1})	325 ± 83	344 ± 70	430 ± 105	263 ± 9	279 ± 36	ns	–
Na (mg kg^{-1})	254 ± 2 ab	268 ± 43 a	271 ± 13 a	159 ± 21 ab	133 ± 14 b	–	*
P (mg kg^{-1})	18 ± 5 b	23 ± 1 b	62 ± 2 a* **	58 ± 3 a* **	49 ± 9 a* **	* **	–
Crop yield							
Melon Yield (kg ha^{-1})	–	15,093 ± 298 b	26,272 ± 3329 a* *	20,287 ± 3038 b	24,759 ± 2050 a* *	* *	–
Number of melons (num ha^{-1})	–	5548 ± 46 b	7752 ± 140 ab	7395 ± 39 ab	8455 ± 547 a	–	*
Cowpea Yield (kg ha^{-1})	2053 ± 59 a	–	106 ± 39 b	871 ± 82 c	463 ± 60 d	* **	–

(mean±sd; n = 5). In each cultivation system (*, **, ***) represent significant differences with respect to the melon monocrop system (control treatment) by Dunnett's test (*P < 0.05; **P < 0.01, **P < 0.001, respectively); missing asterisks denote non-significant differences. Different letters represent significant differences between systems by Tukey's test or Dunn's Kruskal-Wallis Multiple Comparison test; EC, Electrical conductivity; TOC, Total organic carbon; TN, Total nitrogen; NH4 + total ammonium Ca, Mg, K, Na and P; available Ca, Mg, K, Na and P; C, Cowpea monocrop; M, Melon monocrop; MC1, mixed intercropping; MC2, row intercropping 1:1; MC3, row intercropping 2:1.

higher in the monocrop system than in the intercropping systems (Table 1).

3.2. Effects of intercropping on bacterial community diversity and community structure

After filtering, 821,795 reads were yielded and 6676 OTUs were identified with 97% similarity for the bacterial community. No signifi- cant differences were found in the Shannon or Chao1 diversity indexes between cropping systems (Figure S1).

Bacterial community structures were distinctly grouped by cropping system on a PCoA plot (Fig. 5). Moreover, the bacterial community structure in the monocrop systems (M and C) differed significantly (F = 2.7262; P = 0.001) from that in the intercropping systems (MC1, MC2 and MC3). This difference was confirmed by pairwise comparison (Table S1).

3.3. Effects of intercropping on soil bacterial composition

Sequence analyses at the phylum and genus taxonomic levels are shown in Fig. 2A and B. Proteobacteria was the most abundant phylum (40%), followed by Actinobacteria (31%). It is noticeable that

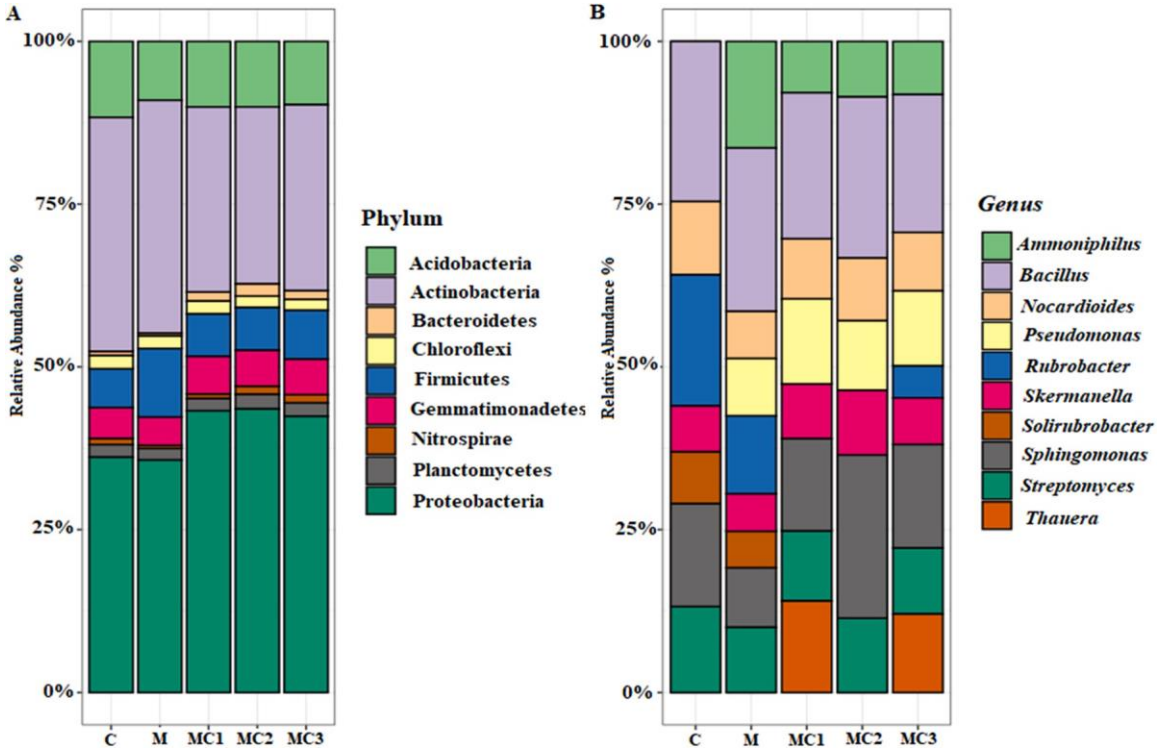

Fig. 2. Relative abundance (>1%) at (A) phylum and (B) genus level of soil bacterial community of intercropping systems. Barplot represents the average of samples for each taxon in each cropping system (n = 5). C, cowpea monocrop; M, melon monocrop; MC1, mixed intercropping; MC2, row intercropping 1:1; MC3, row intercropping 2:1.

Proteobacteria and Bacteroidetes were significantly more abundant and Actinobacteria significantly less abundant in the intercropped soil systems (MC1, MC2 and MC3) than in the monocrop soils (M and C) (Table S2). The other dominant phyla were Acidobacteria (10%), Fir- micutes (7%), Gemmatimonadetes (5%), Planctomycetes (2%), Chlor- oflexi (2%), Bacteroidetes (1%) and Nitrospirae (1%), none of which showed significantly different abundances between the monoculture and intercropping systems (Table S2; Fig. 2A).

The most abundant genera in the different cropping systems were *Bacillus* (23.6%), *Sphingomonas* (17.8%), *Streptomyces* (12.0%), *Nocardioides* (10.1%), *Pseudomonas* (9.0%), *Ammoniphilus* (6.2%), *Rubrobacter* (6.0%), *Skermanella* (5.4%), *Thauera* (4.0%) and *Solirubrobacter* (3.5%) (Fig. 2B; Table S3). *Pseudomonas* was significantly higher in the intercropped systems (MC1, MC2 and MC3) than in the monocrop systems (C and M), whereas *Rubrobacter* and *Solirubrobacter* were significantly lower (Table S3). *Sphingomonas* and *Skermanella* were significantly more abundant in MC2, *Thaurera* in MC1 and *Ammoniphilus* in M.

LDA effect size analysis revealed 20 predominant genera in the melon monocrop (M): *Blastococcus*, *Geofermatophilus*, *Kribella, Kine- ococcus, Actinoplanes, Micromonospora, Actinophytocola, Saccha- romonospora*, *Nonomuraea*, *Actinomadura*, *Rubrobacter*, *Gaiella, Parviterribacter, Solirubacter, Tumebacillus, Gemmatimonas, Microvirda, Rubellimicrobium, Vulcaniibacterium* and *Opitutus.* In the cowpea mono- crop (C), on the other hand, only four genera were predominant: *Pseu- donocardia*, *Hyphomicrobium*, *Methylotenera* and *Phaselicystis.* In the intercropped systems, five genera were selected as predominant in MC1 (*Peptoclostridium, Turicibacter, Amphiplicatus, Ralstonia* and *Stenotrophomonas*); one genus was predominant in MC2 (*Leptolyngbya*); and one genus was predominant in MC3 (*Piscinibacter*) (Fig. 3; Table S4).

3.4. Effects of intercropping on abundance of genes involved in soil N cycling

Concerning the specific gene community related to N cycles, strong differences were found in AMOA, NARG ($P < 0.05$) and NIRK ($P < 0.01$) genes. In general, the log copies of these three genes were higher in monocropping systems (M and C) than in intercropped systems (MC1, MC2 and MC3). Among the three intercropping patterns, MC2 showed the lowest values (Fig. 4; Table S5).

3.5. Effect of intercropping on the soil properties and enzyme activities

Significant differences were found in some of the physicochemical and chemical soil properties (Table 1). Compared to the melon monocrop (M), TN was significantly ($p < 0.05$) higher in all the intercropped systems, MC1, MC2 and MC3 (with an increase of 18% each compared with monocropping). NH_4^+ was also higher in all the intercropped systems assayed than in the monocrops, but it was only significantly higher for the MC3 treatment ($p < 0.05$). The TOC content was also higher in intercropped systems, MC1, MC2 and MC3 (with an increase of

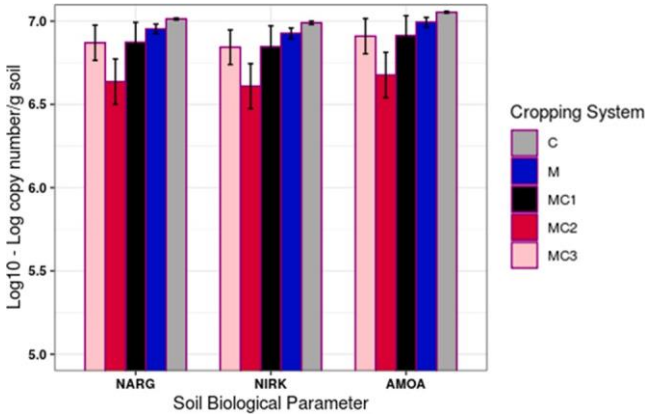

Fig. 4. Influence of intercropping on abundance of NARG, NIRK and AMOA genes belong to soil N cycle. (Bars represent means±sd; n = 5); C, cowpeamonocrop; M, melon monocrop; MC1, mixed intercropping; MC2, row intercropping 1:1; MC3, row intercropping 2:1; NARG, narG gene; NIRK, nirK gene; AMOA, amoA gene.

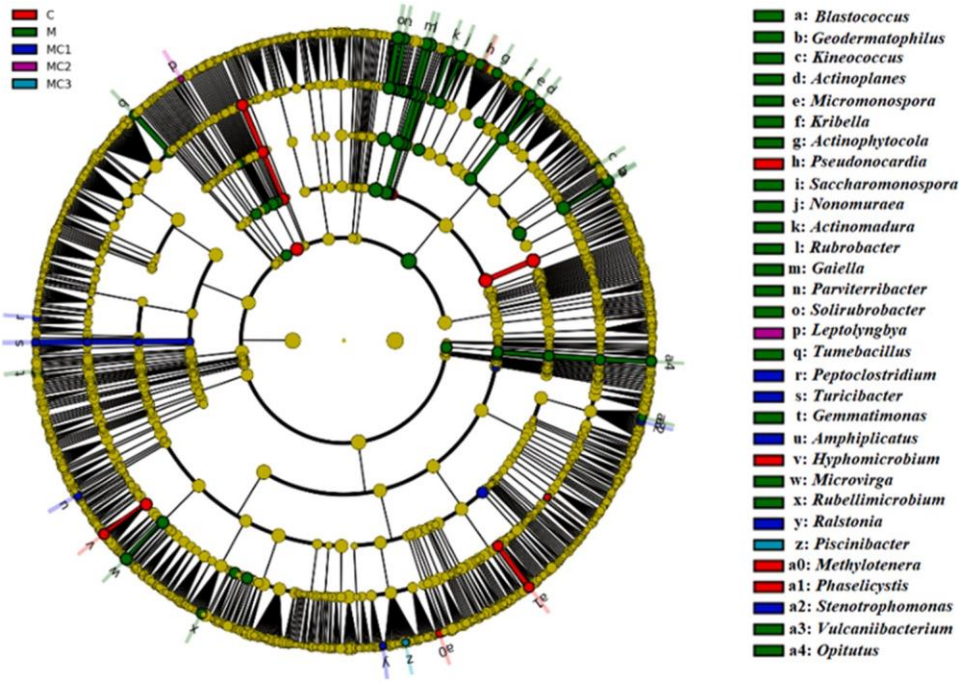

Fig. 3. Cladogram indicating the polygenetic distribution of bacterial lineages at genus level in the intercropping systems as determined by linear discriminant analysis (LDA) effect size (LEfSe). Each circle's diameter is proportional to the taxon's abundance. C, cowpea monocrop; M, melon monocrop; MC1, mixed inter- cropping; MC2, row intercropping 1:1; MC3, row intercropping 2:1.

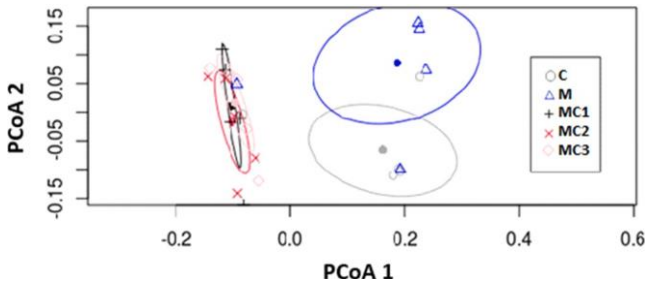

Fig. 5. Principal Coordinate Analysis (PCoA) of bacterial distributions in different intercropping systems. PCoA displays group centroids and dispersions. C, cowpea monocrop; M, melon monocrop; MC1, mixed intercropping; MC2, row intercropping 1:1; MC3, row intercropping 2:1.

18% MC1 and MC2 and 25% MC3 compared with monocropping) compared to the melon monocrop (M). MC3 and C showed the highest TOC content. The available P content was significantly higher ($p < 0.001$) in the intercropped systems [MC1 (169%), MC2 (152%) and MC3 (113%)] than in both monocrops (M and C). Available Mg and Na were significantly higher ($p < 0.05$) in MC1 than in the other treat- ments, and available Ca was significantly higher in MC1, M and C. No significant differences were observed in available K (Table 1).

Soil enzyme activities after intercropping are shown in Table S6. No significant differences were observed in dehydrogenase activity after one year of intercropping compared to the monocrops. Phosphatase activity, on the other hand, showed a significant increase in MC2 (12%) compared to M, while such differences were not observed in MC1 and MC3. β-glucosidase activity increased in MC1 (50%), MC2 (18%) andMC3 (13%) compared to the melon monocrop (M).

3.6. Relationships between soil properties and the bacterial community

Redundancy analysis (RDA) (Fig. 6) revealed a relationship between the bacterial community structure, soil properties and crop yield. TheTN, AmoA, available Na and P and melon yield appeared to be strongly correlated with the bacterial community. Namely, the TN, P content and melon crop yield were correlated with the intercropped systems, while AmoA and Na content were correlated with monocrops. Intercropping systems showed clear divergence from the monocrops (M) and (C), while the latter could not be easily separated.

Significant correlation was observed between *Pseudomonas* and the chemical and harvest parameters of the intercropping systems: for P, TN and melon yield, the correlations were r 0.69, $P < 0.01$; r 0.70, $P < 0.01$; and r 0.68, $P < 0.05$, respectively.

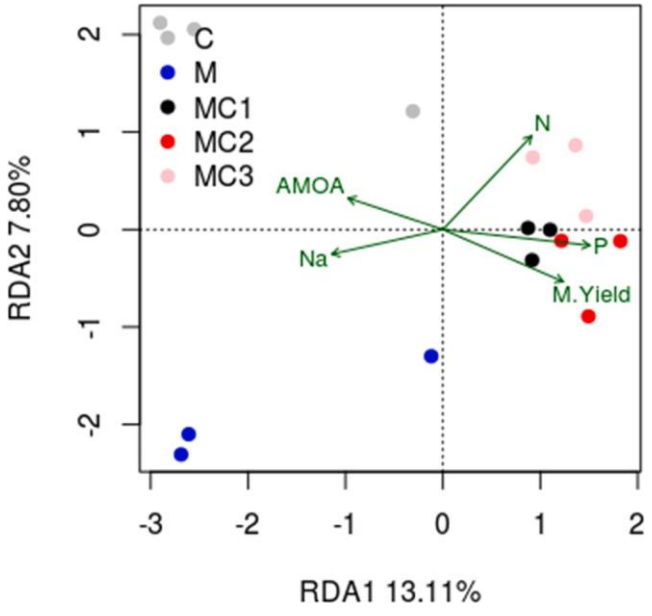

Fig. 6. Redundancy analysis (RDA) based on bacterial community composition of intercropping systems. Sites are coloured by cropping system whereas vectors show the correlation of the chemical, biological and harvest parameters with the community. Na, P, available Na, P; N, total nitrogen; M. Yield, melon yield; C, cowpea monocrop; M, melon monocrop; MC1, mixed intercropping; MC2,row intercropping 1:1; MC3, row intercropping 2:1.

4. Discussion

Intercropping is considered to be an environmentally friendly system that can improve crop yield as well as water and nutrient-use efficiency (Chen et al., 2018; Gaiser et al., 2004). Crops have different needs, so it is especially important to combine them in the right way to obtain yield improvements. As far as we know, the melon-cowpea intercropping system and intercropping patterns between these two crops have not been studied in depth. However, this combination could be an important choice for sustainable horticulture management. The cowpea is a legume, which fixes atmospheric nitrogen and thus supplies it to companion plants like watermelon or other melons that at the same time provide soil shading to conserve water moisture (Munisse et al., 2012).

This study indicated that intercropping melon/cowpea in the first year of experimentation changed the microenvironment and altered the soil nutrient content. These changes positively affected soil microbial community growth, soil microbial community structure and crop yield, which ameliorated the problems associated with monocrops. The intercropping systems assayed (MC1, MC2 and MC3) increased melon yield (34% 74%) with respect to the melon monocrop (M), even though 30% less fertilization was used in the intercropped systems. This increase in yield could be due to higher nitrogen disposal from the cowpea rhizosphere, which should be higher in soils with low N fertilization addition (Yu et al., 2018). This fact has previously been observed in other cowpea intercrop relationships, such as cowpea-maize (Latatiet al., 2014), cowpea-sorghum (Oseni, 2010) and cowpea-cassava (Sikirou and Wydra, 2008). The cropping patterns and N fertilizationrates can alter soil conditions, which subsequently influence the abun- dance of functional N-cycling genes (Tatti et al., 2014). In our study, we also observed a decreasing trend in nitrification and denitrification processes in the three intercropped systems compared to the monocrops.This decrease in the intercropping systems could allow for sustainable nutrient use, diminishing nitrate loss due to leaching and N oxide emissions (Yang et al., 2018).

The results showed that the intercropped soil improved TN content, available P and phosphatase and β-glucosidase enzyme activities compared to the melon monocrop (M), probably due to the melon/ cowpea rhizosphere microorganisms. The normal physiological activ- ities of those microorganisms promote biochemical reactions in the soil microenvironment by secreting extracellular enzymes and releasing intracellular enzymes into the soil (Zeng et al., 2020). In general, legume crops included in intercropping systems improve P availability and soil organic carbon (Ngwira et al., 2012), mostly through root exudates, nodules, and the sloughing off of root cells and root turnover during the growing season (Namatsheve et al., 2020). Roots excrete larger amounts of protons and carboxylates (malonate, malate, and citrate), which would facilitate root-borne phosphatases to hydrolyze organic P (Hin- singer et al., 2011). According to Zhang et al. (2017). Organic P hy- drolysis is also likely supported by a high abundance of phosphate-solubilizing bacteria like *Pseudomonas*, which were more abundant in the intercropped soils and correlated with available P, TN and melon yield. Moreover, the presence of several phosphate-solubilizing bacteria like *Bacillus* in both the monocrops and intercropping systems could also influence in this behavior, previously observed by Chen et al. (2006) and Panhwar et al. (2014).

It is important to note that soil microbial community composition is

significantly correlated with changes in soil chemical properties (Campbell et al., 2010; Lauber et al., 2008). In this study, the TN content, available P, AmoA abundance and melon crop yield play important roles in changes in the microbial community structure. Our findings could indicate that nutrient changes subsequently affect the carbon- and nitrogen-use efficiency of bacteria. Generally, an increase in soil microbial diversity is beneficial to soil function and health, but no differences were detected through diversity or richness estimators, indicating that our hypothesis was not validated. To date, there has been no consensus about changes in alpha diversity caused by intercropping systems, since some researchers have reported that some intercropping systems can increase diversity (Zhang et al., 2015; Zhou et al., 2011), while others have found no significant changes (FU et al., 2019; Poggio, 2005).

In our study, we found significant differences in the bacterial community structure between intercropping and monocrop systems, although not between the different intercropping patterns. These differences showed the influence of cowpea on the bacterial structure of the melon crop, suggesting that cowpea could play an important role in maintaining agricultural ecosystem stability and improving crop growth (P. Li et al., 2018). The differences also suggest that interspecies interactions may affect the abundance of some soil microbial populations, but not population diversity (Z.-M. Li et al., 2018; Yu et al., 2019). The dominant taxonomic groups identified in the soils assayed were Proteobacteria, Actinobacteria, Acidobacteria, Firmicutes, Gemmatimonadetes, Planctomycetes, Chloroflexi, Bacteroidetes and Nitrospirae, all depicted as common inhabitants of soil (Zhou et al., 2018). A higher relative abundance of Proteobacteria and Bacteriodetes and a lower abundance of Actinobacteria in the intercropping systems than in the monocrop systems indicated that both plant species and planting patterns can change the abundance of dominant bacterial phyla (FU et al., 2019; Gong et al., 2019; Zhang et al., 2018) due to their adaptability to a new microenvironment. Moreover, Bacteroidetes were associated with N and P soil cycling (Lidbury et al., 2021), and several plant-beneficial microorganisms identified as *Pseudomonas*, *Bacillus, Streptomyces* and *Sphingomonas* (Asaf et al., 2020; Bhattacharyya and Jha, 2012) could reduce the proportion of harmful fungi (Negawo and Beyene, 2017) due to their suppressive activity and their plant promoting growth (Sivasakthi et al., 2014; Tejera-Hernández et al., 2011).

LEfSe analysis indicated which microorganisms are significantly associated with the different cropping systems. The largest number of bacteria were found in the melon monocrop (*Blastococcus, Geodermatophilus, Kineococcus, Actinoplanes, Kribella* or *Gemmatimonas),* and these bacteria have been described as drought-resistant microorganisms (Castro et al., 2018a, 2018b). On the other hand, only five bacteria were associated with the intercropping systems, which indicates that changes are occurring, despite the high resilience of the bacterial community to changes (Griffiths and Philippot, 2013). Moreover, these changes do not depend too much on the specific intercropping pattern. These results indicate that one year of intercropping, which has been studied here, is not enough to result in certain significant microorganisms. It would be expected that long-term intercropping in the same soils would significantly increase the microbial diversity and its function on soils.

5. Conclusion

The intercropping system produced bacterial community structure changes, which correlated with an increase in soil TN and P concentrations and melon crop yield. The intercropped systems were characterized by a higher abundance of beneficial microorganisms such as *Pseudomonas, Bacillus, Streptomyces and Sphingomonas*. In this first-year experiment intercropping cowpea with melon resulted in a sustainable cropping system using less external input and resulting in an increase in melon yield. Starting from the first year, the use of diversified cropping systems thus provides a regular supply of food and income. Further long-term analysis of these intercropping systems will be needed to reinforce findings on the positive interaction between cowpea and melon microbiota and their functions and to study more in depth which intercrop- ping pattern would be the most beneficial for the farmer.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agee.2022.107856.

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Supplementary Material of A first year melon/cowpea intercropping system improve soil nutrients and change microbial community

A first year melon/cowpea intercropping system improve soil nutrients and change microbial community.

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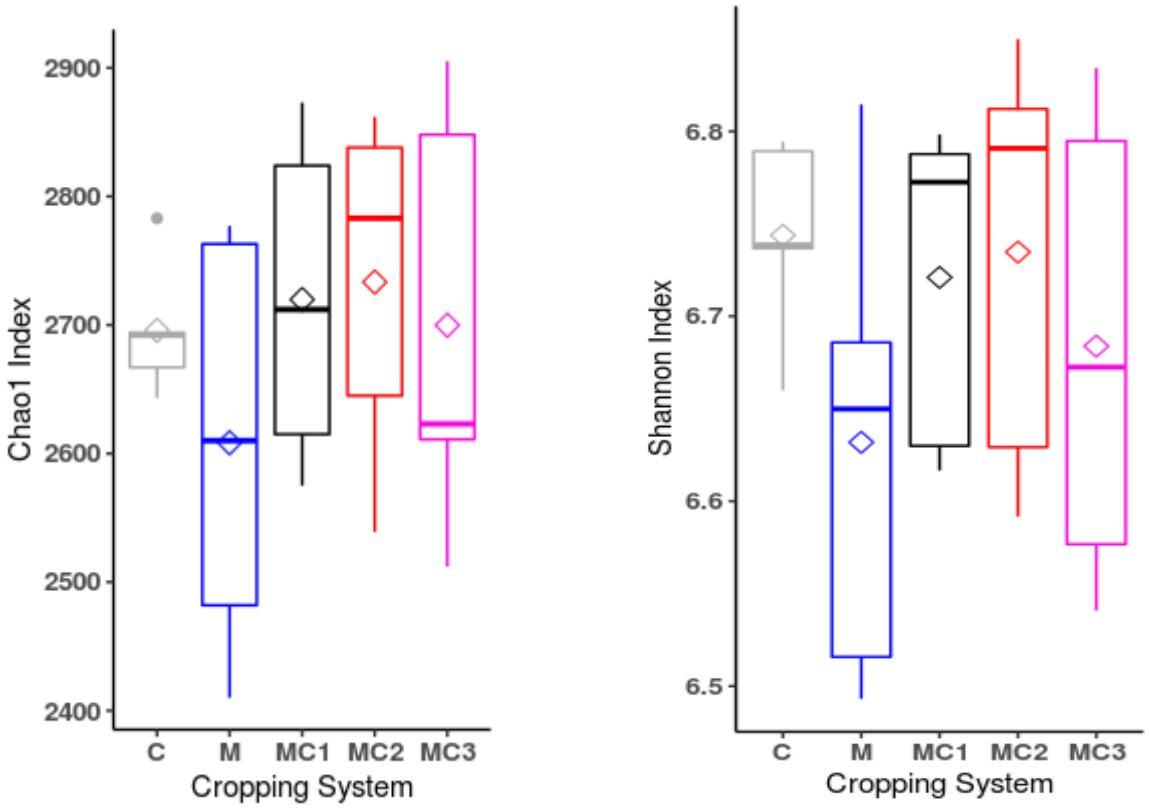

Supplementary Figure 1. Effect of different intercropping systems in bacteria alpha diversity indices.

Chao1 (left) reflects OTU soil bacterial abundance, Shannon (right) reflects OTU soil bacterial diversity. Boxes represent interquartile range (IQR) between first and third quartiles (which correspond with 25th and 75th percentiles, respectively). Horizontal line in the box defines the median and diamond the mean (n=5). Whiskers correspond to the lowest and highest values (1.5 times the IQR from 25th and 75th percentiles) whereas "•" correspond to values higher than 1.5 times and less than three times the IQR; C, cowpea monocrop; M, melon monocrop; MC1, mixed intercropping; MC2, row intercropping 1:1; MC3, row intercropping 2:1.

Supplementary Table 1. Pairwise comparations of bacterial community between different intercropping systems using Bray-Curtis distance.

Pairs	F.Model	R2	(P value)	(P adjusted)	
M vs C	1.083992	0.1193299	0.462	0.4620	ns
M vs MC1	4.028663	0.3349219	0.024	0.0336	*
M vs MC2	4.198942	0.3442054	0.012	0.0336	*
M vs MC3	3.747973	0.3190314	0.029	0.0338	*
C vs MC1	3.916626	0.3286690	0.024	0.0336	*
C vs MC2	3.913404	0.3284875	0.023	0.0336	*
C vs MC3	3.704734	0.3165159	0.024	0.0336	*

Significant levels: (*** p < 0.001; **, p< 0.01; *, p < 0.05); ns, no significant differences;
C, cowpea monocrop; M, melon monocrop; MC1, mixed intercropping; MC2, row intercropping 1:1; MC3, row intercropping 2:1.

Supplementary Table 2. Relative abundance of bacteria at the phylum level (>1%) from intercropping soils

Phylum	C	M	MC1	MC2	MC3	Anova	Kruskal-Wallis
Proteobacteria	36.13±4.22 bc	35.70±4.40 c	43.27±2.52 ab*	43.53±3.77 a*	42.42±3.94 ac*	**	-
Actinobacteria	35.93±5.25 a	35.75±5.55 a	28.43±1.55 ab	27.15±3.22 b*	28.54±5.01 ab	**	-
Acidobacteria	11.67±2.18	9.03±2.52	10.06±2.66	10.05±2.73	9.73±0.69	ns	-
Firmicutes	5.94±2.24	10.53±4.33	6.50±2.10	6.52±2.12	7.45±2.05	ns	-
Gemmatimonadetes	4.72±0.83	4.35±0.53	5.80±1.18	5.53±0.76	5.50±1.13	ns	-
Planctomycetes	1.94±0.29	1.79±0.21	1.86±0.35	2.24±0.53	2.05±0.23	ns	-
Chloroflexi	2.04±0.16	1.91±0.13	1.98±0.18	1.77±0.25	1.69±0.36	ns	-
Bacteroidetes	0.67±0.62 b	0.47±0.64 b	1.41±0.35 ab	1.91±0.74 a	1.36±0.21 ab	-	**
Nitrospirae	0.96±0.55	0.47±0.64	0.73±0.67	1.28±0.19	1.26±0.12	-	ns

(mean±sd; n=5). In each cultivation system (*, **, ***) represent significant differences with respect to the melon monocrop system (control treatment) by Dunnett's test (*P < 0.05; **P < 0.01, **P < 0.001, respectively); missing asterisks denote non-significant differences. Different letters represent significant differences between systems by Tukey's test or Dunn's Kruskal-Wallis Multiple Comparison test; C, cowpea monocrop; M, melon monocrop; MC1, mixed intercropping; MC2, row intercropping 1:1; MC3, row intercropping 2:1.

Supplementary Table 3. Relative abundance of bacteria at the genus level (>1%) from intercropping soils

Genus	C	M	MC1	MC2	MC3	Anova	Kruskal-Wallis
Bacillus	24.57±5.00	25.04±2.80	22.43±2.29	24.70±1.83	21.16±4.35	ns	-
Sphingomonas	15.78±5.41 ab	9.15±1.15 b	14.15±3.93 ab	25.07±2.22 a	15.89±2.99 ab	-	**
Rubrobacter	20.09±1.80 a	11.96±2.81 a	0.00±0.00 b	0.00±0.00 b	4.94±0.47 ab	-	***
Pseudomonas	0.00±0.00 b	8.80±0.97 ab	13.05±2.89 a	10.64±2.49 a	11.48±2.31 a	-	*
Ammoniphilus	0.00±0.00 b	16.38±3.21 a	7.87±2.21 ab	8.53±2.15 ab	8.17±2.23 ab	-	***
Streptomyces	13.19±1.96	9.98±1.61	10.77±1.53	11.40±2.39	10.14±2.49	ns	-
Nocardioides	11.29±0.94a	7.29±1.56 ab	7.25±1.15 b	9.68±1.30 b	9.00±2.57 b		*
Thauera	0.00±0.00 b	0.00±0.00 b	14.04±0.81 a	0.00±0.00 b	12.05±2.99 a	-	***
Solirubrobacter	7.96±1.28 a	5.62±0.85 a	0.00±0.00 b	0.00±0.00 b	0.00±0.00 b	-	***
Skermanella	7.12±0.99 cb	5.77±0.93 c	8.42±0.94 ab***	9.97±0.39 a***	7.16±1.10 bc	***	-

(mean±sd; n=5). In each cultivation system (*, **, ***) represent significant differences with respect to the melon monocrop system (control treatment) by Dunnett's test (*P < 0.05; **P < 0.01, **P < 0.001, respectively); missing asterisks denote non-significant differences. Different letters represent significant differences between systems by Tukey's test or Dunn's Kruskal-Wallis Multiple Comparison test; C, cowpea monocrop; M, melon monocrop; MC1, mixed intercropping; MC2, row intercropping 1:1; MC3, row intercropping 2:1.

Supplementary Table 4. Abundance of significant genus obtained by LefSe analysis in the intercropping systems.

Genus	C	M	MC1	MC2	MC3	LDA		
Blastococcus	$10*10^{-3}±2*10^{-3}$	$9*10^{-3}±2*10^{-3}$	$4*10^{-3}±1*10^{-3}$	$5*10^{-3}±1*10^{-3}$	$5*10^{-3}±1*10^{-3}$	3.07	M	**
Geodermatophilus	$3*10^{-3}±2*10^{-3}$	$3*10^{-3}±2*10^{-3}$	$5*10^{-4}±1*10^{-4}$	$5*10^{-4}±2*10^{-4}$	$5*10^{-4}±2*10^{-4}$	2.64	M	**
Kineococcus	0.00±0.00	$4*10^{-5}±2*10^{-5}$	0.00±0.00	0.00±0.00	0.00±0.00	2.10	M	*
Actinoplanes	$7*10^{-4}±2*10^{-3}$	$8*10^{-4}±2*10^{-4}$	$4*10^{-4}±2*10^{-4}$	$4*10^{-4}±2*10^{-4}$	$4*10^{-4}±2*10^{-4}$	2.07	M	*
Micromonospora	$3*10^{-3}±1*10^{-3}$	$4*10^{-3}±1*10^{-3}$	$2*10^{-3}±1*10^{-3}$	$2*10^{-3}±3*10^{-4}$	$2*10^{-3}±1*10^{-3}$	2.71	M	*
Kribbella	$1*10^{-3}±4*10^{-4}$	$1*10^{-3}±3*10^{-4}$	$1*10^{-3}±4*10^{-4}$	$7*10^{-4}±1*10^{-4}$	$7*10^{-4}±6*10^{-4}$	2.30	M	**
Actinophytocola	$2*10^{-3}±5*10^{-4}$	$1*10^{-3}±5*10^{-4}$	$8*10^{-4}±3*10^{-4}$	$5*10^{-4}±3*10^{-4}$	$6*10^{-4}±3*10^{-4}$	2.29	M	**
Pseudonocardia	$7*10^{-3}±7*10^{-4}$	$6*10^{-3}±6*10^{-4}$	$5*10^{-3}±2*10^{-3}$	$*10^{-4}±3*10^{-4}$	$6*10^{-4}±3*10^{-4}$	2.91	C	*
Saccharomonospora	$1*10^{-3}±6*10^{-4}$	$7*10^{-4}±5*10^{-4}$	$2*10^{-4}±8*10^{-5}$	$8*10^{-5}±4*10^{-5}$	$2*10^{-4}±10*10^{-5}$	2.20	M	**
Nonomuraea	$8*10^{-4}±1*10^{-3}$	$3*10^{-2}±1*10^{-4}$	$1*10^{-3}±6*10^{-4}$	$9*10^{-5}±3*10^{-4}$	$1*10^{-3}±4*10^{-4}$	2.71	M	**
Actinomadura	$5*10^{-4}±4*10^{-4}$	$6*10^{-4}±2*10^{-4}$	$3*10^{-4}±2*10^{-4}$	$2*10^{-4}±7*10^{-5}$	$3*10^{-4}±2*10^{-4}$	2.02	M	**
Rubrobacter	$3*10^{-2}±1*10^{-2}$	$3*10^{-2}±1*10^{-2}$	$7*10^{-3}±1*10^{-3}$	$7*10^{-3}±1*10^{-3}$	$1*10^{-2}±2*10^{-2}$	3.61	M	**
Gaiella	$6*10^{-3}±2*10^{-3}$	$7*10^{-3}±1*10^{-3}$	$5*10^{-3}±6*10^{-4}$	$5*10^{-3}±8*10^{-4}$	$5*10^{-3}±1*10^{-3}$	2.97	M	*
Parviterribacter	$1*10^{-3}±5*10^{-4}$	$1*10^{-3}±7*10^{-4}$	$3*10^{-4}±1*10^{-4}$	$3*10^{-4}±1*10^{-4}$	$3*10^{-4}±8*10^{-5}$	2.30	M	**
Solirubrobacter	$1*10^{-2}±3*10^{-3}$	$1*10^{-2}±3*10^{-3}$	$9*10^{-3}±2*10^{-3}$	$7*10^{-3}±2*10^{-3}$	$9*10^{-3}±1*10^{-3}$	3.20	M	*
Leptolyngbya	0.00±0.00	0.00±0.00	0.00±0.00	$4*10^{-5}±2*10^{-5}$	0.00±0.00	3.04	MC2	*
Tumebacillus	$8*10^{-4}±5*10^{-4}$	$10*10^{-4}±4*10^{-4}$	$2*10^{-4}±9*10^{-5}$	$2*10^{-4}±1*10^{-4}$	$4*10^{-4}±2*10^{-4}$	2.13	M	*
Peptoclostridium	0.00±0.00	0.00±0.00	$4*10^{-5}±6*10^{-5}$	$4*10^{-5}±9*10^{-5}$	0.00±0.00	2.32	MC1	*
Turicibacter	0.00±0.00	0.00±0.00	$8*10^{-5}±4*10^{-5}$	$1*10^{-4}±1*10^{-4}$	$4*10^{-5}±1*10^{-4}$	2.08	MC1	*
Gemmatimonas	$1*10^{-3}±5*10^{-4}$	$2*10^{-3}±5*10^{-4}$	$6*10^{-4}±2*10^{-4}$	$5*10^{-4}±2*10^{-4}$	$6*10^{-4}±3*10^{-4}$	2.36	M	**
Amphiplicatus	0.00±0.00	0.00±0.00	$2*10^{-4}±6*10^{-5}$	$8*10^{-5}±3*10^{-5}$	0.00±0.00	2.15	MC1	**
Hyphomicrobium	$1*10^{-3}±2*10^{-4}$	$7*10^{-4}±6*10^{-4}$	$2*10^{-3}±4*10^{-4}$	$1*10^{-4}±3*10^{-4}$	$1*10^{-3}±3*10^{-4}$	2.08	C	*
Microvirga	$5*10^{-3}±1*10^{-3}$	$7*10^{-3}±3*10^{-3}$	$4*10^{-3}±2*10^{-3}$	$3*10^{-3}±1*10^{-3}$	$3*10^{-3}±1*10^{-3}$	2.92	M	*
Rubellimicrobium	$3*10^{-4}±2*10^{-4}$	$8*10^{-4}±10*10^{-4}$	$1*10^{-4}±1*10^{-4}$	$8*10^{-5}±9*10^{-5}$	$1*10^{-4}±2*10^{-5}$	2.05	M	**
Ralstonia	0.00±0.00	0.00±0.00	$8*10^{-5}±5*10^{-5}$	0.00±0.00	0.00±0.00	2.56	MC1	*
Piscinibacter	0.00±0.00	0.00±0.00	$4*10^{-5}±10*10^{-5}$	$4*10^{-4}±4*10^{-4}$	$4*10^{-5}±1*10^{-5}$	2.38	MC3	*
Methylotenera	$4*10^{-5}±4*10^{-5}$	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	2.16	C	**
Phaselicystis	$1*10^{-3}±5*10^{-4}$	$1*10^{-3}±2*10^{-4}$	$9*10^{-4}±3*10^{-4}$	$6*10^{-4}±1*10^{-4}$	$8*10^{-4}±9*10^{-5}$	2.22	C	*
Stenotrophomonas	0.00±0.00	0.00±0.00	$9*10^{-5}±4*10^{-5}$	$7*10^{-5}±3*10^{-5}$	0.00±0.00	2.33	MC1	*
Vulcaniibacterium	0.00±0.00	$4*10^{-5}±5*10^{-5}$	0.00±0.00	0.00±0.00	0.00±0.00	2.00	M	*
Opitutus	$1*10^{-3}±7*10^{-4}$	$1*10^{-3}±5*10^{-4}$	$1*10^{-4}±2*10^{-4}$	$9*10^{-4}±5*10^{-4}$	$1*10^{-3}±5*10^{-4}$	2.31	M	*

Significant levels: (*** p < 0.001; **, p< 0.01; *, p < 0.05); LDA; Linear Discriminant Analysis value;
C, cowpea monocrop; M, melon monocrop; MC1, mixed intercropping; MC2, row intercropping 1:1; MC3, row intercropping 2:1.

Supplementary Table 5. Different N cycle gen abundance on different intercropping systems.

Soil biological	C	M	MC1	MC2	MC3	Anova	Kruskal-Wallis
AMOA (Log copies of AmoA g soil^{-1})	7.1±0.0 a	7.0±0.0 a	6.9±0.1 ab	6.7±0.1 b	6.9±0.1 ab	-	*
NIRK (Log copies of nirK g soil^{-1})	7.0±0.0 a	6.9±0.0 a	6.8±0.1 ab	6.6±0.1 b**	6.8±0.1 ab	**	-
NARG (Log copies of narG g soil^{-1})	7.0±0.0 a	7.0±0.0 a	6.9±0.1 ab	6.6±0.1 b	6.9±0.1 ab	-	*

(mean±sd; n=5). In each cultivation system (*, **, ***) represent significant differences with respect to the melon monocrop system (control treatment) by Dunnett's test (*P < 0.05; **P < 0.01, **P < 0.001, respectively); missing asterisks denote non-significant differences. Different letters represent significant differences between systems by Tukey's test or Dunn's Kruskal-Wallis Multiple Comparison test; C, cowpea monocrop; M, melon monocrop; MC1, mixed intercropping; MC2, row intercropping 1:1; MC3, row intercropping 2:1.

Supplementary Table 6. Different enzyme activity on different intercropping systems.

Enzyme	C	M	MC1	MC2	MC3	Anova	Kruskal-Wallis
Beta-Glucosidase (nmol INTF g soil^{-1} per hour)	243±80 b	198±23.9 b	337±49.9 a***	249±43.6 ab	284±27.8 ab*	**	-
Phosphatase (nmol INTF g soil^{-1} per hour)	81±14.3 b	111±7.07 a	138±39.3 a	154±15.8 a	83.6±23.3 b	-	*
Dehydrogenase (nmol INTF g soil^{-1} per hour)	25.1±3.96	31.1±1.56	28.1±6.47	28.2±4.40	33.8±6.98	-	ns

(mean±sd; n=5). In each cultivation system (*, **, ***) represent significant differences with respect to the melon monocrop system (control treatment) by Dunnett's test (*P < 0.05; **P < 0.01, **P < 0.001, respectively); missing asterisks denote non-significant differences. Different letters represent significant differences between systems by Tukey's test or Dunn's Kruskal-Wallis Multiple Comparison test; C, cowpea monocrop; M, melon monocrop; MC1, mixed intercropping; MC2, row intercropping 1:1; MC3, row intercropping 2:1

Conclusions

- Sustainable agriculture management demonstrates a soil improvement, reflected by changes in bacterial and fungal communities, in addition to an increase on nutritional soil properties. Furthermore, sustainable agriculture yields are maintained or even increased in comparison to conventional agriculture management; fact that can be asserted that sustainable agriculture can reduce the use of fertilizers, pesticides, herbicides and favoured the soil health.

- The *univariate statistical* analysis identified those variables on which the cropping system had a greater impact, such as the increased of nitrogen, carbon, phosphorus, ammonium and micronutrients and the significant differences in abundance of different phyla and genera whereas the *multivariate statistical* analysis detected differences in soil microbial diversity between conventional and sustainable managements and provides global overviews about the relationship both of microbial community and cultivation system (respectively) with physical, physicochemical and chemical soil properties.

- The use of bioinformatic tools such as *Linear Discriminant Analysis Effect Size* (LEfSe) and *Molecular Ecological Network Analysis* (MENA) allow to determine the most affected taxa as well as the topological structure of the microbial network for each cultivation system. Compost application promotes a more modular and complex bacterial network than conventional that allows the bacterial community to have a higher capacity to respond to environmental changes.

- The application of prediction algorithms such as *Phylogenetic Investigation of Communities Reconstruction of Unobserved States* (PICRUSt) verify how the compost amendment contributes to an increase of nitrogen fixing bacteria that could have also contributed to the improvement of soil quality. This could be due to changes on bacterial habitats that could have also altered bacterial functions, showing higher predicted nitrogen-fixing potential, decreased N_2O emissions and greater carbon-sequestration potential than conventional or manure soil management. This implies the importance of using a stable organic amendment as compost and the use of adequate tools to study the response of the bacterial community to long-term cropping systems.

- The application of *univariate*, *multivariate* statistical techniques and *bioinformatic* algorithms by computarional resources have also allowed evaluating the changes that occur when intercropping system is assayed, showing an increase of beneficial microorganisms such as *Pseudomonas*, *Bacillus*, *Sphingomonas* and, *Streptomyces* but also showing how soil properties improve, increasing especially total organic carbon and phosphorous.

The support of different bioinformatic and statistical techniques allows a global and specific study of complex environments such the soil; therefore, it can be concluded that a precise bioinformatic and statistical approach is crucial to get a global vision but also concrete of the studied sample.

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Appendix

Paper 1: Changes in Bacterial and Fungal Soil Communities in Long-Term Organic Cropping Systems

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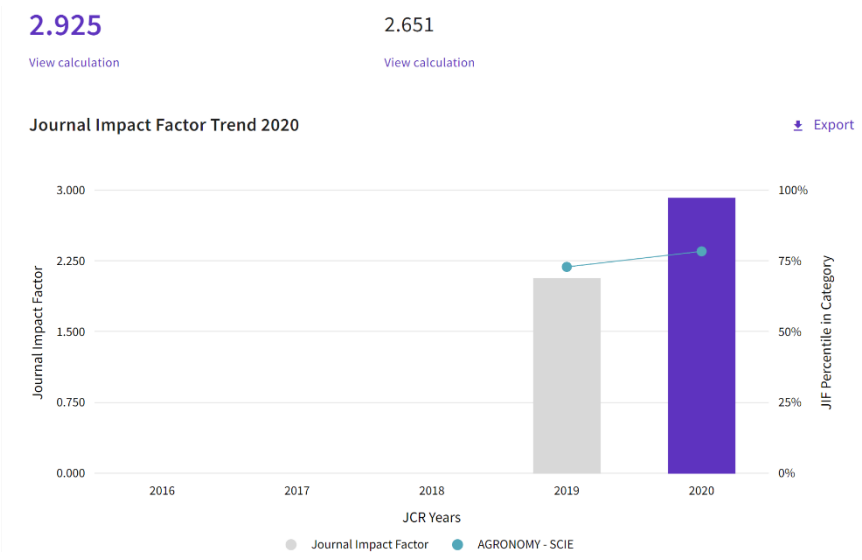

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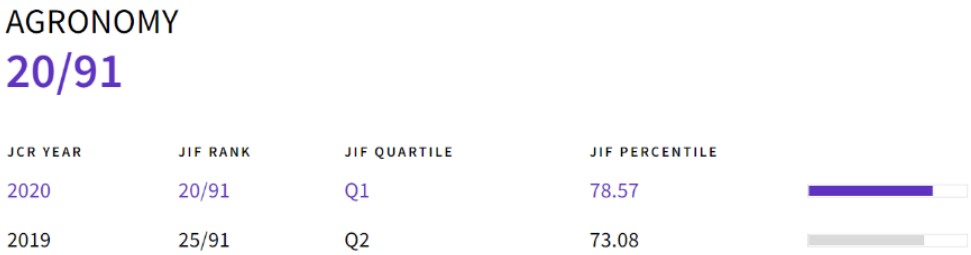

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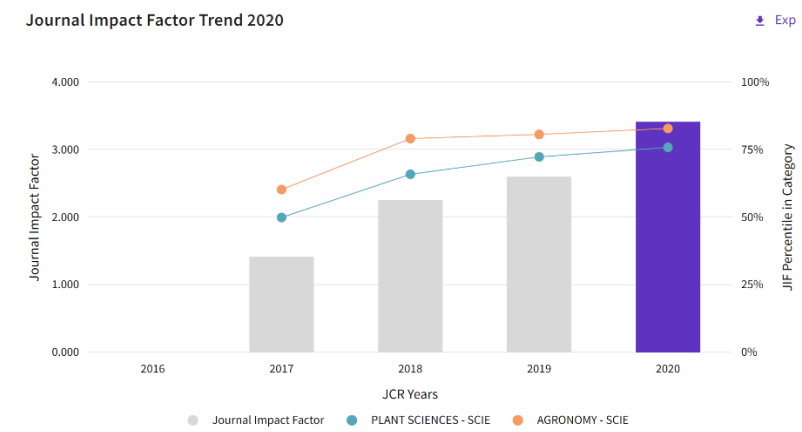

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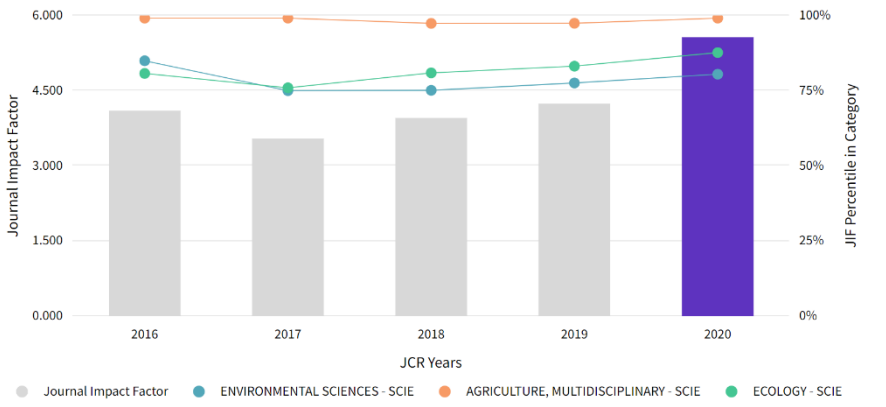

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2017	1/57	Q1	99.12
2016	1/56	Q1	99.11