

RESEARCH ARTICLE

A metagenomic approach in the evaluation of the soil microbiome in coffee plantations under organic and conventional production in tropical agroecosystems

Alejandra Cabrera-Rodríguez¹, Ricardo Trejo-Calzada¹, Cristina García-De la Peña²,
Jesús G. Arreola-Ávila¹, Erika Nava-Reyna^{3*}, Felipe Vaca-Paniagua⁴, Clara Díaz-Velásquez⁴,
César A. Meza-Herrera¹

¹Universidad Autónoma Chapingo-URUZA, Carretera Gómez Palacio-Ciudad Juárez Km. 40, s/n, 35230 Bermejillo, Durango, México,

²Facultad de Ciencias Biológicas, Universidad Juárez del Estado de Durango, Av. Universidad s/n, Fracc. Filadelfia, 35010 Gómez Palacio, Durango, México, ³CENID-RASPA-INIFAP, Km. 6.5 Margen derecha Canal Sacramento. C.P. 35140. Gómez Palacio, Durango, México,

⁴Laboratorio Nacional en Salud: Diagnóstico Molecular y Efecto Ambiental en Enfermedades Crónico-Degenerativas, Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México, Av. De Los Barrios 1, Los Reyes Iztacala, 54090 Tlalnepantla, Estado de México

ABSTRACT

The aim of this study was to determine the soil microbiome throughout mass sequencing in coffee plantations managed with either an organic (OAM; i.e., bio-fertilizers *Azospirillum brasilense* and *Glomus intraradices*) or a conventional (CAM; i.e., traditional NPK-fertilization) agronomic systems. Soil microbiome samples were collected in tropical eastern Mexico (Veracruz, 19°28' N & 96° 52' W), with annual average temperature and rainfall of 24.8° C, and 882.6 mm, respectively. Upon DNA soil-microbiome extraction, the V3-V4 16S rRNA region was amplified, and sequenced (Illumina). Results were analyzed with QIIME based on the EzBioCloud reference. Diverse phyla (n = 16), classes (n = 40), orders (n = 90), families (n = 135) and genera (n = 333) were identified. The diversity index values were similar in both treatments, with Shannon's being 9.7 and Simpson's 0.99. While the phylum Proteobacteria was more abundant in CAM-soils and classified as copiotrophic, the phylum Acidobacteria was more abundant in OAM-soils and classified as oligotrophic. This classification may be related to the application of microorganisms and their effect on the soil's state of organic matter and carbon fractions. Our research outcomes indicate that the application of bio-fertilizers promoted an increased presence of Acidobacteria, a phylum positively correlated with organic matter while significantly involved in carbon sequestration. Undisputable, metagenomics emerges as an interesting up-to-date genomic technology for unveiling the hidden content of the soil microbiome black box.

Keywords: 16S rRNA gene; Microorganisms; Copiotrophic; Oligotrophic; Bio-fertilizer

INTRODUCTION

Microorganisms (MOS) are the living component of the soil; their abundance and metabolic activity depend mainly on the soil type and management, plant growth, as well as root exudates. Certainly, MOS activity influences the availability of essential elements for the plants in that they participate in the decomposition of organic matter (Schinner, 2012; Singh et al., 2011). In this regard, a lack of nutrients in the soil, triggered by different conventional agronomic practices, modifies the composition, structure, and activity of microbial communities (Hernández et al., 2013). This modification

avoids the soil from acting as a substrate in that organic fractions are altered, the last being directly proportional to the amount of organic carbon in soil (Bastida et al., 2015).

Diverse management practices in agricultural production systems can modify the soil microbiome properties, as well as increase the microbial biomass content (Diacono and Montemurro, 2011). One of these practices is bio-fertilization, in which probiotics are used because of their ability, through biological processes, to mobilize nutrients in the soil and make them available to plants (Ritika and Utpal, 2014). These probiotics also promote plant growth and yield, which in turn has a positive effect

*Corresponding author:

Erika Nava-Reyna, CENID-RASPA-INIFAP, Km. 6.5 Margen derecha Canal Sacramento. C.P. 35140. Gómez Palacio, Durango, México.
E-mail: nava.erika@inifap.gob.mx

Received: 05 January 2020; Accepted: 11 April 2020

on humification of organic matter and conservation of microbial structures (Cotler et al., 2016; Ortega et al., 2016). Therefore, it is important to consider this type of organic practice in crops of economic interest, since in these crops conventional practices are usually employed, and they can affect the microbial structure of soil and, consequently, its fertility.

One of the most economically and culturally important crops worldwide is coffee, with a harvesting area of 10.6 million ha and a production of 10.3 million tons. In Mexico, coffee (*Coffea arabica* L.) is also an important crop, harvested in more than 630,000 ha, a production of 158,323 tons while 250 million USD economic value. Nonetheless, from the total planted coffee area in Mexico, only 3.24% is classified as organic production; Chiapas, Veracruz and Puebla are the main producer's states of both conventional and organic coffee, the latter standing out because of its high demand by North American and European markets. Coffee cultivation constitutes 0.66% of the national agricultural gross domestic product, with an annual per capita consumption of 0.6 kg (Flores, 2015; SAGARPA, 2017 and 2018).

Because of such economic importance, diverse agricultural management practices have been proposed to maximize coffee production through the implementation of organic-based production systems to promote the recovery of the soil's optimal status (SAGARPA, 2017). For these reasons, is important to identify and analyze the presence of microorganism communities in soils under organic production systems where organic practices are being implemented to conserve and enhance biotic resources available in coffee plantations (Hernández et al., 2018). Besides, it is fundamental to know the main processes that affect the soil fertility, such as the presence and dynamics of soil microorganisms (Barea et al., 2005; Carbonetto et al., 2014). Certainly, a better understanding of the dominant bacterial taxa in the soil will improve our understanding regarding the main MOS functional capabilities (Delgado et al., 2018).

Nowadays, metagenomics is a tool for the study of bacterial communities used to determine, explore, and analyze microbial communities from diverse environments, through the sequencing of their genetic material (Cadena et al., 2016; Ospino et al., 2018).

Previous studies have been carried out on coffee plantations to determine how the chemical and biological properties of soil are modified in response to organic or conventional management systems. Some of the most evaluated properties include pH, electrical conductivity, nitrogen, phosphorus, microbial biomass carbon,

enzymatic activity, microbial respiration, and bacterial colony forming units (Chemura, 2014; Lammel et al., 2015; Velmourougane, 2016). Similarly, some studies have focused on the isolation and identification of bacteria in plant and rhizosphere organs, using various methodologies. These studies have confirmed the presence of endophytic bacteria in seeds, leaves, stems and roots, some of which have been isolated to evaluate the inhibition of disease-promoting bacteria (Vega et al., 2005; Shiomi et al., 2006). Other studies have been addressed on identifying the bacteria in coffee cherries through the polymerase chain reaction of the 16S rRNA gene (Silva et al., 2000; Vilela et al., 2010; Oliveira et al., 2013). In addition, the role of various rhizobacteria as nitrogen fixers (Jimenez-Salgado et al., 1997), their potential to synthesize acetic acid and to degrade ethanol precursors (Muleta et al., 2009), as well as their importance as phosphate solubilizers (Muleta et al., 2013) have also been studied.

Nonetheless, little is currently known about the response of soil bacterial communities to the type of management in coffee plantations, using the metagenomics approach in order to get a more accurate picture of the soil-microbiome structure and composition. Based on previous findings, we hypothesized that organic management, through the application of bio-fertilizers, could enhance the presence of bacteria involved in carbon sequestration (i.e. acidobacteria), and then, plant growth. Therefore, we determined the soil microbiome in coffee plantations managed under organic or conventional production systems, through massive 16S rRNA sequencing. Secondary objectives included: 1) to determine the bacterial structure and composition in soils, and 2) to establish the main changes in bacterial structure in soils under organic and conventional management.

MATERIALS AND METHODS

Location and environmental conditions of the study area

Soil-microbiome samples were collected in the State of Veracruz with an annual average temperature and rainfall of 24.8° C, and 882.6 mm, respectively (Díaz et al., 2006). The samples were collected from coffee plantations under organic (OAM) or conventional (CAM) agricultural management located in the municipality of Emiliano Zapata, Veracruz, Mexico (19°28'0.98" North, and 96°52'38.20" West; Figs.1 and 2).

Treatments groups, soil microbiome sampling and samples management

The organic agronomic system (OAS treatment) corresponds to coffee plantations whose soil was treated

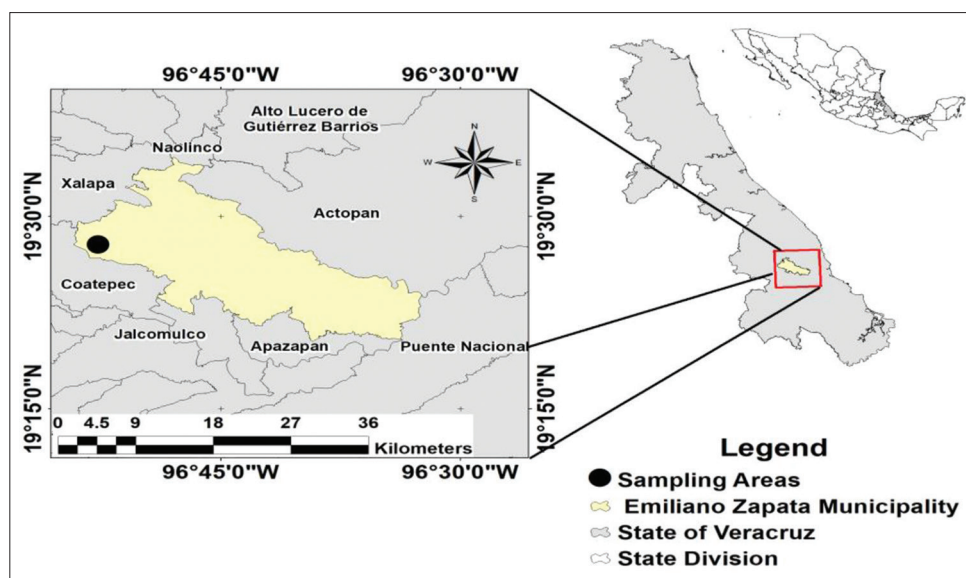


Fig 1. Geographical location of the soil microbiome samples from coffee plantations either under organic (OAM) or conventional (CAM) agronomic systems in eastern tropical Mexico (Veracruz; 19° N, 96° W).



Fig 2. (A) Plants of *Coffea arabica* located in the study area. (B) Soil microbiome sampling in coffee plantations either under organic (OAM) or conventional (CAM) agronomic systems in eastern tropical Mexico (Veracruz; 19° N, 96° W).

with bio-fertilizer application (i.e. *Azospirillum brasilense* and *Glomus intraradice*; Biofábrica Siglo XXI®) during a five year period. In the OAS-treatment, two annual doses of bio-fertilizer were added, one at the beginning of the rainy season and the other three weeks later, at a concentration of 5x10¹¹ Colony Forming Units of *A. brasilense* and 9x10⁴ spores of *G. intraradices*.

The control group corresponds to the conventional agronomic system (CAS treatment) where the soil's coffee plantations were treated with two to three chemical applications of conventional fertilizers (16:16:16 and 17:7:14 NPK) per year were applied aligned to the rainy season. In both treatments groups (i.e. OAS and CAS), 0.25 g of soil was taken in the rhizosphere area at 10 cm deep. Each soil sample was placed in a Zymo Research™ BashingBead™ tube for cell lysis, then 740 µl of lysis/stabilization solution were added, and finally each tube

was processed in a TerraLyzer™ cell disruptor for 30 s; samples were kept at room temperature.

DNA extraction from the soil's microbiome and bioinformatics analyses

Soil microbiome DNA was extracted using the Zymo Research™ Xpeditio™ Zymo BIOMICSTM kit in a laminar UV flow hood under sterile conditions and following the manufacturer's instructions. Briefly, the soil microbiome DNA was extracted on a 1.2% agarose gel at 80V for 45 min in a BIORAD electrophoresis chamber (Bio-Rad Laboratories, Inc.) in order to visualize the presence of high molecular weight DNA. The visualization was carried out in a GelMax™ photo documenter (UVP LLC). The amount of DNA obtained was measured in a Qubit™ fluorometer (Invitrogen). Amplification of the V3-V4 region of the 16S rRNA gene was carried out according to Klindworth et al. (2013): S-D-Bact-0341-b-S-17, 5'-CCTACGGGNGGCWGCAG-3' and S-D-Bact-0785-a-A-21, 5' GACTACHVGGGTATCTA ATCC-3', which produce an amplicon of ~460 bp. The sequences were synthesized with the "overhang" adapters of the Illumina protocol (2017a), being as follows: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3' and 5'-GTCTCGT GGGCTCGGAGATGT GTA TAA GAGACAGGACTACHVGGGTATCTAATCC-3' (amplicon of ~550 bp).

The Illumina PCR protocol (2017a) was used, the amplicons were purified with 0.8% Agencourt® AMPure® XP beads, and then labeled using the Nextera XT Index Kit™ for library preparation, following the Illumina

protocol (2017b). Finally, quantification, normalization, library clustering and next-generation mass sequencing were performed following the 16S metagenomic protocol (Illumina, 2017a). The sequences were analyzed in the Oracle VM VirtualBox 5.1.14 on the MGLinux platform using Quantitative Insights Into Microbial Ecology bioinformatics software (QIIME) v.1.9.0 (Caporaso et al., 2010), as previously suggested (García-De la Peña et al., 2019). The number of sequences was plotted by the number of taxa at the genus level to observe the coverage depth (PAST ver 3.15, Hammer et al., 2001). Using the KRONA program (Ondov et al., 2011), graphs were generated by treatment to visualize the taxonomic levels from phylum to genus. Alpha diversity was determined using the Shannon and Simpson indices.

RESULTS AND DISCUSSION

The arithmetic mean of the total number of sequences obtained for both treatments before assembly was 34,991, whereas the mean of assembled and discarded sequences was 13,791 and 21,200, respectively. On average, 91 chimeras were removed, leaving a mean of 13,700 quality sequences. After taxonomic assignment, a mean of 12,606 bacterial sequences was obtained; however, once the singletons were removed, the final sequences were 5,559. The rarefaction curves for the operational taxonomic units (OTUs) recorded an adequate coverage depth where the samples reaching the asymptote at about 6,000 sequences (Fig. 3). The diversity index values were similar in both treatments, with Shannon's being 9.7 and Simpson's 0.99. In both soil samples, 16 phyla were recorded, of which Proteobacteria (54%), Acidobacteria (22%) and Planctomycetes (7%) were the most abundant in the OAM group (Fig. 4); corresponding values for the CAM treatment were 56%, 16% and 7% (Fig. 5). A total of 40 classes

were determined, among which Alphaproteobacteria, Solibacter and Acidobacteria were the most abundant in both treatments; OAM recorded 53%, 11% and 7% (Fig. 4), while CAM recorded 56%, 5% and 3%, respectively (Fig. 5).

From the 90 orders obtained, Rhizobiales, Rhodospirales and Sphingomonadales showed the highest percentages; corresponding values for the OAM were 23%, 20% and 7%, respectively (Fig. 4), with corresponding values of 28%, 16% and 10%, for the CAM group. (Fig. 5). From the 135 recorded families, Rhodospirillaceae (19%), Bradyrhizobiaceae (16%) and Sphingomonadaceae (7%) predominated in the OAM group (Fig. 4), whereas the CAM treatment obtained corresponding values of 15%, 14% and 9%, respectively (Fig. 5).

In both treatments, a total of 333 genera were reported. The most abundant were *Pseudolabrys* (6%), *Rhodoplanes* (5%) and *Solibacter* (6%) in the OAM (Fig. 4), with corresponding values of 6%, 3%, and 2%, in the CAM group (Fig. 5). Proteobacteria was 2% more abundant in CAM than in OAM, while Acidobacteria was 6% more abundant in OAM, with no difference in Planctomycetes abundance.

The working hypothesis stated that in the production of organic coffee, the application of bio-fertilizers would promote an increased presence of acidobacteria, a phylum positively correlated with organic matter while significantly involved in carbon sequestration. The obtained outcomes of the study support such a hypothesis. The observed soil-bio-fertilizer-biome a scenario in our study suggest increases in the stable fractions of organic matter, making more resistant to decomposition while increasing carbon sequestration in soil, a situation which in at same time, improves plant growth by stimulating root development,

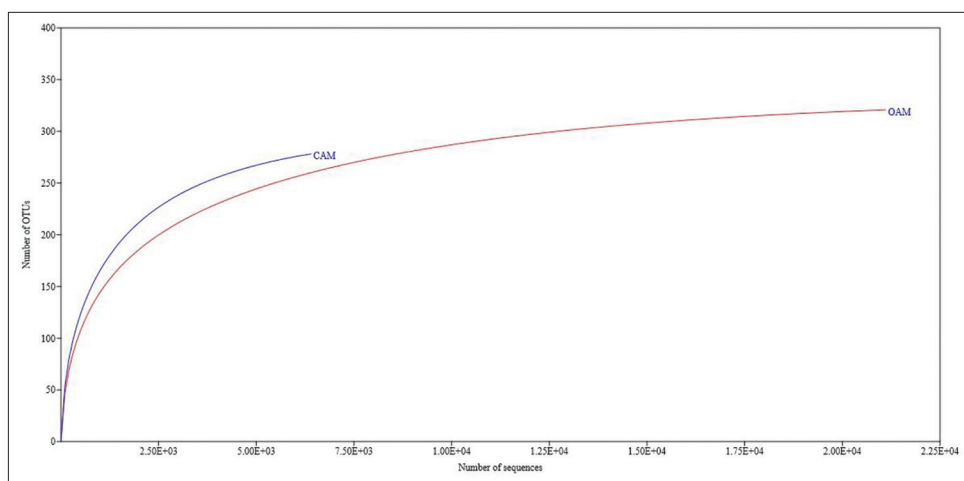


Fig 3. Number of sequences by the number of operational taxonomic units (OTUs) in soil microbiome samples from coffee plantations either under organic (OAM) or conventional (CAM) agronomic systems in eastern tropical Mexico (Veracruz; 19° N, 96° W).

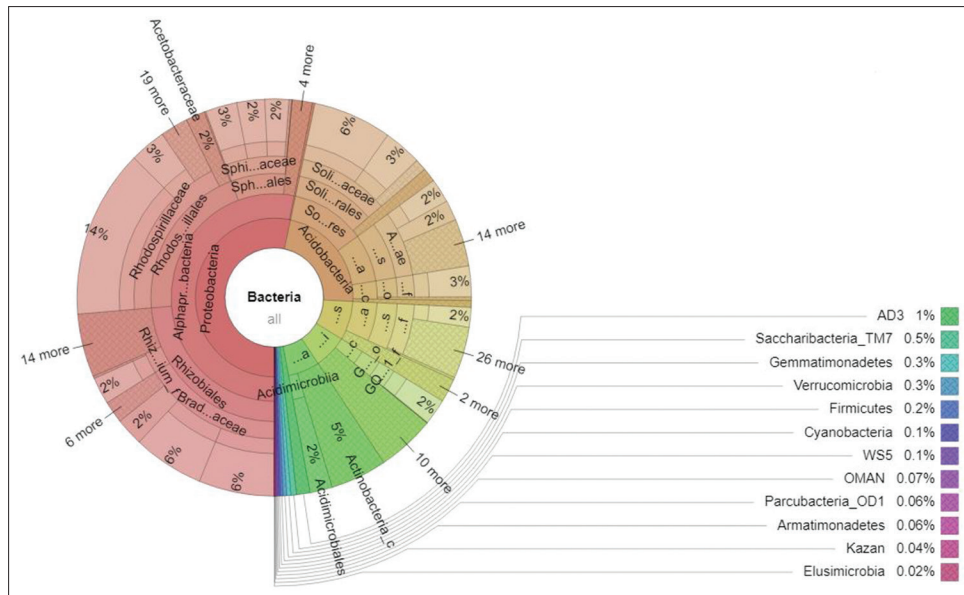


Fig 4. Relative abundance of bacterial taxa found in soil microbiome samples from organic coffee plantations in eastern tropical Mexico (Veracruz; 19° N, 96° W).

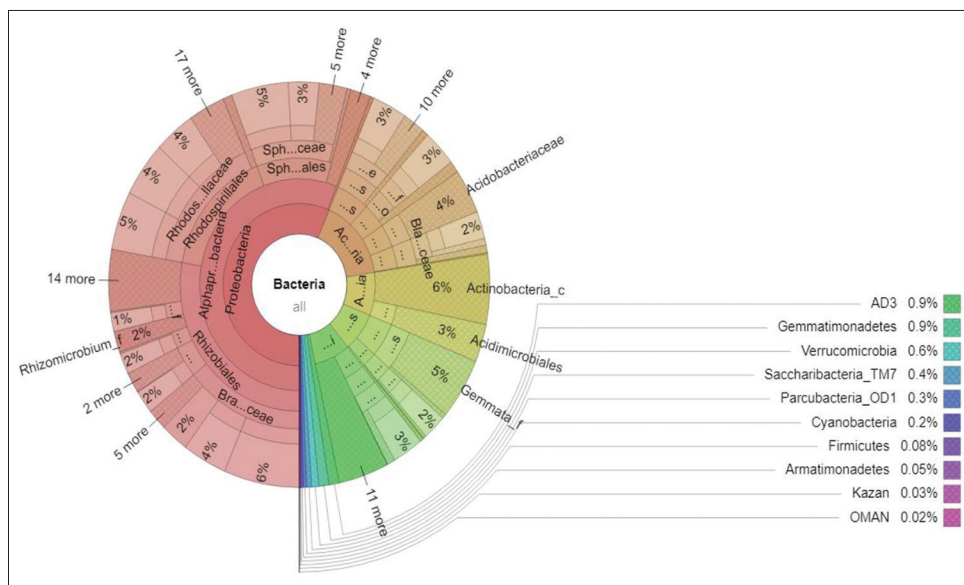


Fig 5. Relative abundance of bacterial taxa found in soil microbiome samples from conventional coffee plantations in eastern tropical Mexico (Veracruz; 19° N, 96° W).

as well as nutrient and water uptake (Dębska et al., 2016; Fatunbi and Ncube, 2009).

Acidobacteria has been reported as one of the most abundant phyla in tropical forests and ecosystems similar to those in the study area (Carbonetto et al., 2014; Delgado et al., 2018). In the same way, the phylum Acidobacteria and Proteobacteria have been grouped as oligotrophic and copiotrophic organisms, respectively (Zhalnina et al., 2014). Most oligotrophic organisms are located in soils with low nutrient availability and a high amount of recalcitrant organic matter. On the other hand, copiotrophic organisms

are abundant in conditions with high available nutritional yields and are capable of consuming labile organic carbon (Carbonetto et al., 2014; Koch, 2001; Vigdis and Øvreås, 2008). Moreover, the enrichment of copiotrophic bacteria has been related to the increase of CO₂ emission (Sheng and Zhu, 2018). On this regard, Fierer et al. (2007) showed that a greater abundance of Acidobacteria occurs in the soils with low mineralization rates; in contrast, the abundance of Proteobacteria was higher in the soils with high carbon availability. Acidobacteria dominates the soils with high organic matter content and is involved in the microbial degradation of lignocellulose plant biomass.

They are also capable of using carbon as an energy source and their distribution is related to the availability of carbon in the soil (Eichorst et al., 2011; Rawat et al., 2012).

Also, members of this phylum, to which the classes Solibacter and Acidobacteria belong, and the genus Solibacter, are essential drivers in ecosystem processes (Kielak et al., 2010; Zhang et al., 2014). In a study by Wang et al. (2020), phylum Acidobacteria was more abundant in soils without agronomic management (23%) than in soils with conventional management (20%). On the other hand, Pan et al. (2014) showed that in grassland soils after applying nitrogen fertilization, there was a positive correlation between the phylum Proteobacteria and different forms of nitrogen (ammonium, nitrate, and nitrite). Otherwise, the most abundant class of pool was Alphaproteobacteria, which is one of the most representative and abundant in arid and tropical ecosystem soils, such as in the study area (Delgado et al., 2018). Besides, Rhizobiales is considered one of the most important orders due to its ability to establish symbiosis with plant roots through rhizobia, which stabilize soil bacterial communities (Martínez et al., 2015).

The genus *Azospirillum* was not the most abundant in both production systems, however, this genus belongs to the order Rhodospirales and to the family Bradyrhizobiaceae, which were more abundant in the OAM group. On this regard, Aguirre et al. (2011) indicated that in coffee crops, the application of *A. brasilense* induced a greater root development with which the plants developed a better anchorage and greater efficiency in the use of both nutrients and water.

Applying bio-fertilizers increases the stable fractions of organic matter, which makes it more resistant to decomposition, and in turn increases carbon sequestration in soil (Dębska et al., 2016; Fatunbi and Ncube, 2009). Such an increase, as well as the augment in the carbon content of microbial biomass, was demonstrated after three years of biofertilizer application based on *Pseudomonas* spp, *Penicillium* and *Actinomyces* spp (Piotrowska et al., 2012). Likewise, biofertilization with *A. brasilense* modifies the microbial communities of rhizosphere under field conditions (García de Salamone, 2012). Furthermore, soil bacterial communities in wheat crops responded to different nitrogen fertilization treatments and bio-fertilization with *A. brasilense* (Di Salvo et al., 2018).

Results of this study suggests that the organic use of bio-fertilizers significantly contributed to the increase in recalcitrant forms of carbon, which in turn, improves plant growth by stimulating root development, as well as nutrient and water uptake. On the other hand, conventional management with NPK-fertilization promotes an increase

in available forms of minerals for microorganisms, but without accumulation of organic matter and particularly of, recalcitrant carbon. This would explain the relatively greater abundance of Acidobacteria in OAM and the greater abundance of Proteobacteria in CAM. It is likely that long-term continuous management (i.e. bio-fertilization) favors an even greater abundance of Acidobacteria in the OAM group. Knowing how soil microbial diversity is involved with the function of any edaphic ecosystem is essential to understand the ecosystem compensatory responses to counteract the threat of a changing environment.

CONCLUSIONS

The metagenomics analyses of the soil microbiome in coffee plantations under organic and conventional production systems allowed the classification of the more abundant taxonomic groups into oligotrophic and copiotrophic organisms, respectively. Even though diversity index values were similar in soils under different OAM and CAM production systems, Proteobacteria was more abundant in CAM, while Acidobacteria was more plentiful in OAM, suggesting that agronomic management influences the structure and function of bacterial communities. Moreover, OAM seems to stimulate the proliferation of certain bacteria that are related to soil carbon sequestration and plant growth. Unquestionable, metagenomics emerges as a remarkable up-to-date genomic technology for the unveiling of the taxonomic content in the soil microbiome. Such an approach would help to determine, analyze, and classify the microorganisms of different agroecosystems in order not only to conserve but to enhance the soil's biotic resources.

ACKNOWLEDGMENTS

This project, entitled “Impact of Biofertilizer Application on Carbon Sequestration and the State of Soil Organic Matter,” was funded by the Ministry of Agriculture and Rural Development. Thanks are also due to Irene Pacheco-Torres for her assistance in the laboratory work, and to the National Council of Science and Technology (CONACYT, Mexico) for its funding contribution.

Author contributions

Study conception and design: E. Nava-Reyna and C. García-De la Peña; Experimental work and data acquisition: A. Cabrera-Rodríguez, C. García-De la Peña, E. Nava-Reyna, F. Vaca-Paniagua and C. Díaz-Velásquez; Analysis of results and bibliographic research: A. Cabrera-Rodríguez; Data interpretation: A. Cabrera-Rodríguez, C. García-De la Peña, E. Nava-Reyna and R. Trejo-Calzada; Manuscript writing: A. Cabrera-Rodríguez; Critical review of the

manuscript: C. García-De la Peña, E. Nava-Reyna, R. Trejo-Calzada, G. Arreola-Ávila and C. Meza-Herrera; Research supervision; E. Nava-Reyna.

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