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Silver and Hematite Nanoparticles Had a Limited Effect on the Bacterial Community Structure in Soil Cultivated with *Phaseolus vulgaris* L.

Karla E. Zarco-González [1], Jessica D. Valle-García [1], Yendi E. Navarro-Noya [2], Fabián Fernández-Luqueño [3] and Luc Dendooven [1,*]

[1] Laboratory of Soil Ecology, CINVESTAV, Mexico City 07360, Mexico; estephaniazg.130897@gmail.com (K.E.Z.-G.); jd.valleg@gmail.com (J.D.V.-G.)
[2] Laboratory of Biotic Interactions, Center for Biological Science Research, Tlaxcala Autonomous University, Tlaxcala 90120, Mexico; yendiebenezer.navarro.n@uatx.mx
[3] Sustainability of Natural Resources and Energy Programs, CINVESTAV, Saltillo 25900, Mexico; cinves.cp.cha.luqueno@gmail.com
* Correspondence: dendooven@me.com

Abstract: The amount of nanoparticles that enters the environment has increased substantially in the last years. How they might affect plant characteristics and the bacterial community structure when they enter the soil, however, is still debated, as there is a continuous interaction between them. In this study, we determined the effect of silver (Ag-NPs) and hematite (α-Fe_2O_3-NPs) nanoparticles (0.15 g kg^{-1}) on the characteristics of common bean (*Phaseolus vulgaris* L.) and the rhizosphere, non-rhizosphere and uncultivated soil bacterial community. The application of Ag-NPs or α-Fe_2O_3-NPs did not affect plant growth, but changed the amount of some heavy metals in the roots and aerial parts. The application of nanoparticles had a limited effect on the diversity, structure and functional profile of the soil and rhizosphere bacterial communities, but they were altered by cultivation of the bean plants and changed over time. It was found that application of Ag-NPs or α-Fe_2O_3-NPs had no effect on bean plant growth and only a small effect on the bacterial community structure and its putative metabolic functions. These findings show that in a complex system, such as a soil, different factors might affect the bacterial community structure and alter the possible effect of nanoparticles on it.

Keywords: nanotoxicology; ecotoxicology; soil microbiome; nanomaterials; iron-based nanomaterial

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1. Introduction

Nanoparticles (NPs) are materials with at least one dimension between 1 and 100 nm. They have unique physical and chemical properties, such as a higher surface area due to their size [1]. Their characteristics make them valuable in medicine, catalysis, electronics, agriculture, the textile industry and environmental remediation [2,3]. Nanoparticles are classified into two groups, i.e., organic, such as fullerenes and carbon nanotubes, and inorganic, which can be metal or metal oxide nanoparticles [4].

Some of the most common inorganic nanoparticles contain silver (Ag-NPs), zinc oxide (ZnO-NPs), copper oxide (CuO-NPs), titanium dioxide (TiO_2-NPs) or iron oxides (Fe_2O_3-NPs and Fe_3O_4-NPs) [1]. Silver nanoparticles are extensively used for their antimicrobial activity and are added to personal care products, textiles, paint and food packaging materials [5,6]. Iron oxide nanoparticles are used in medical diagnostics, for the controlled release of drugs and for water remediation processes and have been applied to iron (Fe)-deficient arable soils [7,8].

As the global production of nanoparticles has increased, their presence in the environment has increased, also. Nanoparticles may enter the environment during their production, or when wastewater or biosolid containing them is applied to the soil [4,9,10].

The bioavailability and impact of nanoparticles on soil ecosystems is determined by their characteristics, such as their type, size and concentration, but also by their soil parameters, such as pH, organic matter content and particle size distribution [1,3,5,10,11]. When released in the environment, nanoparticles can accumulate in an ecosystem and might affect plant development. Several studies have reported positive and negative effects on plants due to the application of nanoparticles. Sillen, Thijs, Abbamondi, et al. [12] reported that the biomass of maize (*Zea mays* L.) increased when exposed to Ag-NPs, but Karami Mehrian, Heidari, Rahmani, et al. [13] reported a decrease in the root and shoot length of tomato (*Solanum lycopersicum* L.). Rui, Ma, Hao, et al. [8] reported that the application of Fe_2O_3-NPs increased the biomass, chlorophyll and Fe content of peanut (*Arachis hypogaea* L.) plants.

Soil microorganisms play a key role in biogeochemical cycles and transformation of pollutants [10]; therefore, the effect of different nanoparticles on soil microorganisms has been studied often. On the one hand, Wang, Shu, Zhang, et al. [11] reported that application of Ag-NPs inhibited the metabolic activity of some microorganisms, reduced the abundance of bacteria, and altered the soil microbial community structure. Zhang, Wu, Si, et al. [14] and Chavan and Nadanathangam [6] reported that Ag-NPs were toxic for rhizobacteria. On the other hand, He, Feng, Ren, et al. [7] reported that an application of γ-Fe_2O_3-NPs increased the bacterial abundance and urease and invertase activity. Additionally, iron oxide nanoparticles increased the abundance of ammonia-oxidizing bacteria [15].

The rhizosphere is a dynamic part of the soil environment, where signaling between the plant roots and the soil microbiome occurs [16,17]. Some microorganisms in the rhizosphere can promote plant growth by fixing nitrogen, solubilizing phosphorus, mineralizing organic N and producing siderophores [6]. The continuous interaction between the plant and the soil microbiome can modify the effect that NPs have on both, but the effect might also depend on the type of nanoparticles used and their characteristics. Therefore, bean plants (*Phaseolus vulgaris* L.) were cultivated in soil amended with Ag-NPs and α-Fe_2O_3-NPs to study this interaction while the agronomic parameters, diversity and taxonomic and putative functional profile of the soil bacterial community were determined. The aim of this study was to determine how two distinct nanoparticles, i.e., Ag-NPs and α-Fe_2O_3-NPs, affected:

- the growth and characteristics of common bean plants
- the diversity and taxonomic and putative functional profile of the soil bacterial community in cultivated and uncultivated soil.

2. Materials and Methods

2.1. Characterization of Nanoparticles

The silver and iron oxide nanoparticles (Ag-NPs and α-Fe_2O_3-NPs) were obtained from ID-nano Investigación y Desarrollo de Nanomateriales S.A. de C.V. The nanoparticles were dispersed in deionized water and the size was determined by transmission electron microscopy (TEM) with the JEOL JEM-1400 microscope (JEOL Ltd., Tokyo, Japan) with magnifications ranging from 2500 to 120,000.

2.2. Seeds, Soil Sampling Site and Experimental Design

The seeds of common bean Pinto Saltillo were obtained from Zapata (Arteaga, Coahuila, Mexico). The soil was collected from the Academic Area of Agricultural and Forest Sciences from the Instituto de Ciencias Agropecuarias de la Universidad Autónoma del Estado de Hidalgo (ICAP-UAEH), Tulancingo, Hidalgo (20°03′34.4″ N 98°22′50.7″ W) on 4 June 2019.

Details of the experimental design and characterization are given in Figure S1. Briefly, four different 400 m^2 plots (n = 4) were defined at the sampling site. Soil was collected from the top 0–20 cm layer 10 times and the samples were pooled to obtain a composite sample from each plot. The four soil samples were passed separately through a 5 mm sieve and characterized; details of the methods can be found in Table S1 [18–21]. The field-based replication was maintained throughout the experiment to avoid pseudo-replication [22].

Fifteen subsamples of soil (7 kg each) from each plot (n = 4) were added separately to a polyvinyl chloride (PVC) column (60 cm high × 17 cm diameter) that contained 7 cm "tezontle" (red volcanic rock) (Figure S1). The water content was adjusted to a 40% water holding capacity (WHC) and left to stand for one week. After 7 days, six soil columns from each plot were applied with 0.15 g Ag-NPs kg^{-1} and six with 0.15 g α-Fe_2O_3-NPs kg^{-1}, while the remaining three were left unamended. The nanoparticles were dispersed in 100 mL deionized water, applied to the soil surface and, subsequently, the first 10 cm were mixed. As such, three different treatments were applied to the soil from each plot (n = 4). After three days, three columns with Ag-NPs-amended soil, three with α-Fe_2O_3-NPs-amended soil and the three left unamended were planted with three bean seeds and considered the cultivated soil. The remaining columns were considered the uncultivated soil. One column from each plot (n = 4) and treatment (n = 5) was selected at random, extracted for DNA as described below and considered time zero. All the columns were placed randomly in a greenhouse and watered twice a week for the entire experiment. After 15 days, two plants were removed from the cultivated soil and only one, the most vigorous, was kept in each column. The experiment was conducted in a greenhouse with an average temperature of 25 °C and humidity ranging from 40 to 50%.

After 100 days, the columns were taken (replicates n = 3, treatments n = 5, plots n = 4) and the soil was removed and sampled. Subsamples of 0.5 g rhizosphere (the soil firmly adhered to the root), non-rhizosphere (the rest of the soil volume in the treatments with the plants) and uncultivated soil were taken. All the samples were stored at –20 °C until DNA extraction.

2.3. Measure of Morphological Parameters of the Plant and Mineral Content

The length of the roots and the aerial part of the plant (stem and leaves) was measured and air dried. The samples were ground and sieved at 150 µm and the metal content in the roots and aerial parts was determined. Subsamples of 2 g of plant material were analyzed for copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn) with a PerkinElmer (Waltham, MA, USA) plasma emission spectrometer (Model OPTIMA 8300). The analysis was performed at the Cinvestav-Saltillo chemical analysis laboratory located in Ramos Arizpe, Coahuila, Mexico.

2.4. DNA Extraction

The humic and fulvic acids were removed from the soil samples and cell lysis was achieved using three different methods [23–25]. Protein precipitation and DNA purification was completed following the method described in [26]. Briefly, 1/5 EDTA 0.5 M pH 8 and 1/10 potassium acetate 5 M pH 5 were added to the total volume. The samples were incubated at 4 °C for 30 min and centrifuged at 13,000 rpm and 4 °C for 10 min. The supernatant was transferred to a new tube and extracted with 400 µL chloroform-isoamyl alcohol solution (24:1). The samples were centrifuged at 13,000 rpm at room temperature for 10 min. The aqueous phase was transferred to a new tube and the same procedure was repeated. Polyethylene glycol (PEG) at 13% (w/v) was added and the tubes were incubated at −20 °C overnight. The samples were centrifuged at 13,000 rpm and 4 °C for 10 min, the supernatant was removed, and the DNA pellet was washed with 70% (v/v) cold ethanol. The DNA samples were centrifuged once again under the same conditions, and the excess ethanol was removed and resuspended in 50 µL ultrapure water. The DNA extracted with the three different methods was pooled.

2.5. PCR and DNA Sequencing

The V3–V4 regions of the 16S ribosomal gene were amplified by PCR with the primers 341-F (5′-CCT ACG GGN GGC WGC AG-3′) and 805-R (5′-GAC TAC HVG GGT ATC TAA TCC-3′), which contain the specific adapters of Illumina. The PCR was achieved with the MultiGene™ OptiMax Thermal Cycler (Labnet International, Edison, NJ, USA). The quality of the PCR products was verified by electrophoresis in agarose gel (1% w/v).

The PCR products were purified with the FastGene™ Gel/PCR Extraction kit and quantified with the PicoGreen dsDNA assay on the NanoDropTM 3300 fluorospectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The libraries were sequenced with a 300 paired-end run of the Ilumina MiSeq platform by Macrogene Inc (DNA Sequencing Service, Seoul, Republic of Korea). The raw sequences were deposited in the National Center for Biotechnology Information (NCBI) database Sequence Read Archive (SRA), submission SUB12874223.

2.6. Bioinformatic Analysis

The raw fastq files obtained from the sequencing were processed with "Quantitative Insights Into Microbial Ecology" v 2022.2 (QIIME 2) [27]. The barcode extraction was completed with the extract_barcodes.py function of QIIME v 1.9.1 and the sequences were demultiplexed with QIIME 2. The primer removal, merging of reads, chimera removal, inference of amplicon sequence variants (ASVs), and dereplication were performed with DADA2 in a QIIME2 environment [28]. The filter and trim parameters were -p-trim-left-f 17, -p-trim-left-r 21, -p-trunc-len-f 260 and -p-trunc-len-r 220. The taxonomic assignment of the representative sequences of the ASVs was accomplished with classify-sklearn using a classifier trained against the SILVA reference database (release 138) [29].

The functional profile of the bacterial community was predicted with the "Phylogenetic Investigation of Communities by Reconstruction of Unobserved States" (PICRUSt2) (https://github.com/picrust/picrust2, accessed on 16 November 2022) using the q2-picrust2 plugin v 2021.11_0 of QIIME2. The pathways based on KEGG Orthology (KO) numbers were annotated with the "Kyoto Encyclopedia of Genes and Genomes" (KEGG).

2.7. Statistical Analysis

All the statistical analyses were performed in R v 4.1.1 (R Core Team, used in August 2021) [30]. A one-way ANOVA was used to determine the effect of the nanoparticles on the morphological characteristics and the heavy metal content of the plants. A p value < 0.05 was established to denote a statistically significant effect.

The alpha diversity was determined utilizing Hill numbers using the raw counts dataset [31]. The Hill numbers at $q = 1$ (frequent ASVs) and $q = 2$ (dominant ASVs) were selected because they have a lower bias due to the sampling effort. Hill numbers maintain the same measurement unit across values, i.e., an effective number of species, and can be compared with the conventional diversity indices [31]. A Kruskall–Wallis test was performed to determine the effect of the nanoparticles application or plant cultivation on the alpha diversity, i.e., $q = 1$ and $q = 2$.

The biological observation matrix (BIOM) was imported to R with phyloseq v.1.36.0 to analyze the bacterial communities [32]. The frequency tables were collapsed at the phylum and genus level and the relative abundance was determined with phyloseq v 1.36.0. Barplots were constructed to visualize the relative abundance of the most abundant taxonomic groups in the different treatments.

Subsets of the frequency tables of the taxonomic and functional profiles were used to determine the effect of the application of nanoparticles, the cultivation of *Phaseolus vulgaris* L. or time. The bacterial communities of the rhizosphere and non-rhizospheric soils amended with Ag-NPs and α-Fe_2O_3-NPs were compared with those of the rhizosphere and non-rhizosphere unamended soil to determine the effect of the application of nanoparticles. The rhizosphere and the uncultivated soil amended with the same nanoparticles (Ag-NPs or α-Fe_2O_3-NPs) were compared to determine the effect of common bean cultivation on the bacterial communities. Details of the statistical methods and R packages used can be found in Table S2 [33–38].

3. Results

3.1. Nanoparticles' Characteristics

The TEM micrographs show that the nanoparticles had a quasi-spherical shape. The α-Fe_2O_3-NPs measured between 20–50 nm and the Ag-NPs measured between 5 and 100 nm, but tended to form larger aggregates (Figure 1).

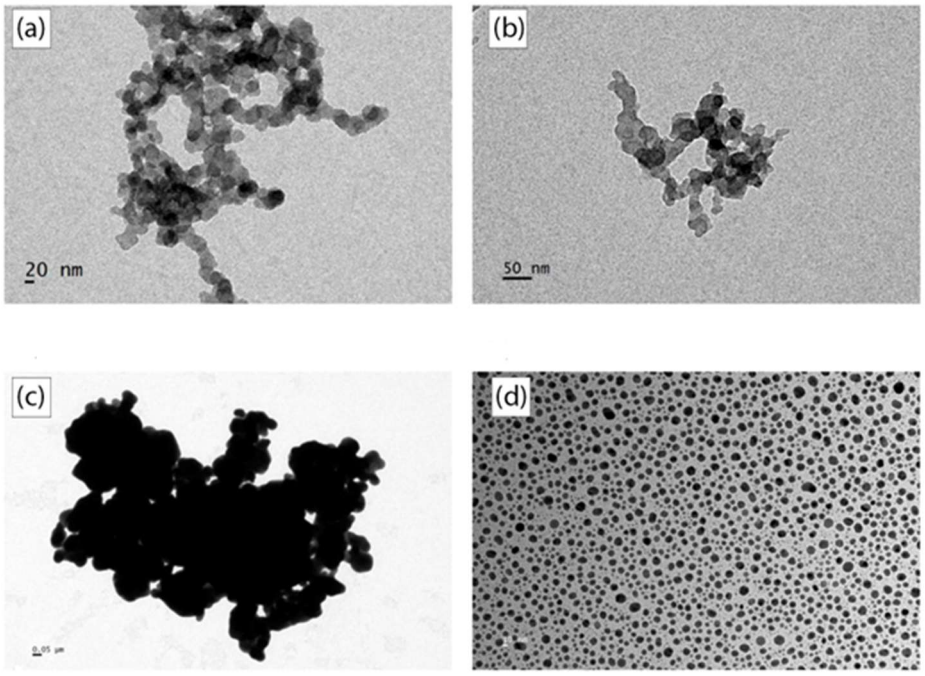


Figure 1. TEM micrographs of α-Fe_2O_3-NPs ((**a**) and (**b**)) and Ag-NPs ((**c**) and (**d**)).

3.2. Soil Characteristics

The clay content of the soil was 340 g kg^{-1}, the sand content 270 g kg^{-1} and the silt content 390 g kg^{-1} on a dry weight basis. The soil had a total C content of 30.7 $\pm$ 4. 1 g C kg^{-1} and a total N content of 2.49 $\pm$ 0.43 g N kg^{-1} on a dry weight basis. The water holding capacity (WHC) was 495 $\pm$ 24 g kg^{-1}, the electrolytic conductivity (EC) 10.69 $\pm$ 3.43 dS m^{-1} and the pH 6.7 $\pm$ 0.1.

3.3. Effect of NPs on Bean Characteristics

The application of nanoparticles had no significant effect on the root and stem length of common bean plants (Table S3). The amount of Cu, Fe, Mn and Zn in the roots of the bean plants cultivated in soil amended with nanoparticles was significantly lower than when cultivated in the unamended soil ($p < 0.05$) (Table S4). The Mn content was significantly lower in the aerial part of plants cultivated in soil amended with Ag-NPs than in the aerial part of plants cultivated in the unamended soil ($p < 0.05$).

3.4. Alpha Diversity and Taxonomic Profile of the Bacterial Community in Soil

A total of 327,263 high-quality sequences were obtained, with an average of 6963 sequences per sample. The sequences were grouped in 2779 ASVs. The application of nanoparticles or the cultivation of beans had no significant effect on the alpha diversity (Figures S2 and S3).

The three most abundant phyla were Proteobacteria (32.2% $\pm$ 1.7%), Actinobacteria (24.1% $\pm$ 2.7%) and Acidobacteria (19.3% $\pm$ 2.7%). Members of Vicinamibacteraceae (3.6% $\pm$ 0.5%), *Defluviicoccus* (3.3% $\pm$ 0.5%) and *Bacillus* (2.5% $\pm$ 0.3%) were the most abundant at the lower bacterial taxonomic levels (Figure 2).

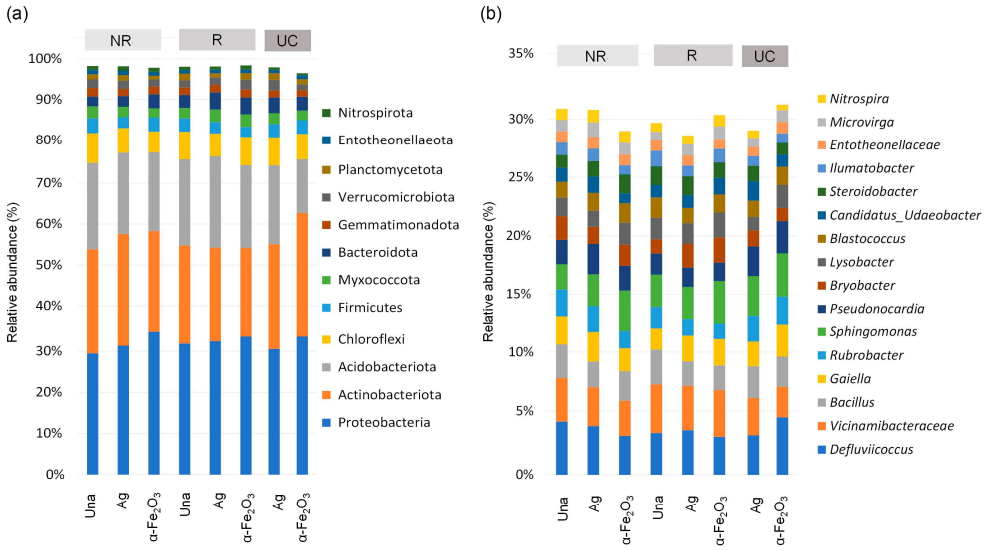
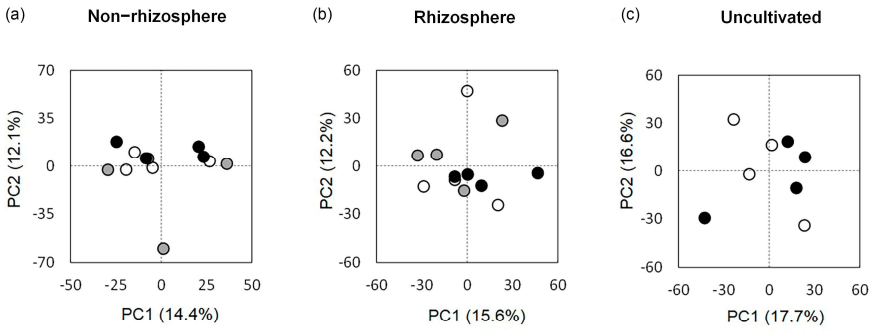


Figure 2. Relative abundance (%) of the most abundant bacterial (**a**) phyla and (**b**) genera in non-rhizosphere soil (NR), the rhizosphere soil (R) of common bean and uncultivated soil (UC) amended with Ag-NPs (Ag), α-Fe_2O_3-NPs (α-Fe_2O_3) or left unamended (Una).

3.5. The Bacterial Community as Affected by Application of the Nanoparticles

The PCA did not separate the bacterial community in the Ag-NPs- and α-Fe_2O_3-NPs-amended, non-rhizosphere and rhizosphere soil from that in the unamended soil (Figure 3). Additionally, the PCA did not separate the bacterial community in the Ag-NPs- and α-Fe_2O_3-NPs-amended from the uncultivated soil. Consequently, the perMANOVA test indicated that the application of nanoparticles had no significant effect on the bacterial community structure. However, the volcano plots showed that the nanoparticles application had a large effect on some bacterial groups (Figure S4). The application of Ag-NPs had a large effect (effect size > |0.8|) on *Rhizobium* and *Bradyrhizobium* in the non-rhizosphere soil and on *Turicibacter* in the rhizosphere. The application of α-Fe_2O_3-NPs had a large effect (effect size > |0.8|) on *Rubrobacter*, *Sphingomonas* and *Stenotrophomonas* in the non-rhizosphere soil, and on *Solirubrobacter* and *Turicibacter* in the rhizosphere (Table S5).



(d)

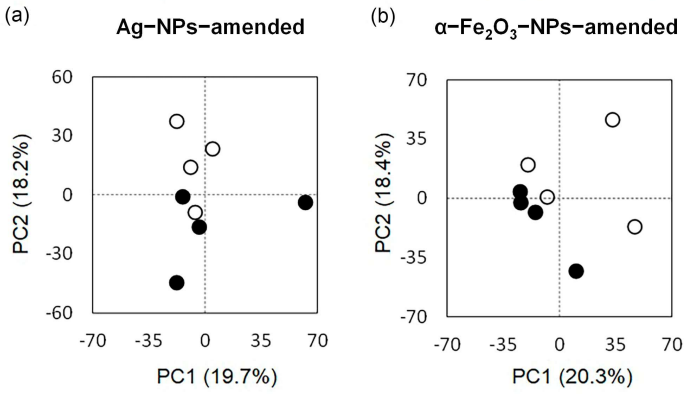
	F	R^2	*p*
Non−rhizosphere	0.961	0.176	0.691
Rhizosphere	0.994	0.181	0.467
Uncultivated	0.947	0.136	0.724

Figure 3. Principal component analysis (PCA) with all bacterial genera in the (**a**) non-rhizosphere, (**b**) rhizosphere of common bean and (**c**) uncultivated soil left unamended (●) or amended with Ag-NPs (○) or α-Fe_2O_3-NPs (●); (**d**) permutational analysis of variance (perMANOVA) test to determine the effect of application of the different nanoparticles on the bacterial community structure at day 100.

The PCA considering the putative bacterial metabolic functions did not separate the Ag-NPs- and α-Fe_2O_3-NPs-amended, non-rhizosphere and rhizosphere soil from the unamended soil (Figure S5). The perMANOVA test showed that the application of nanoparticles did not affect the putative functional profile of the soil bacterial community.

3.6. The Bacterial Community as Affected by Cultivation of the Bean Plants

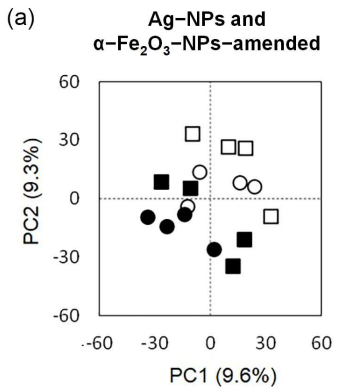
The PCA separated the bacterial community in the rhizosphere from that of the uncultivated soil independent of the type of nanoparticle applied (Figures 4 and 5). The perMANOVA test indicated that cultivation of the beans changed the bacterial community structure significantly ($p < 0.05$) and the dispersion was not significantly different between the rhizosphere and the uncultivated soil (Table S6). The volcano plots show that cultivation of the common bean had a large effect (effect size > |0.8|) on more bacterial groups than the application of nanoparticles (Figure S6).



(c)

	F	R^2	*p*
Ag−NPs−amended	1.190	0.165	0.063
α−Fe_2O_3−NPs−amended	1.227	0.170	0.084

Figure 4. Principal component analysis (PCA) with all bacterial genera in (**a**) Ag-NPs- and (**b**) α-Fe_2O_3-NPs-amended uncultivated soil (○) and rhizosphere soil (●); (**c**) permutational analysis of variance (perMANOVA) test to determine the effect of bean (*Phaseolus vulgaris* L.) cultivation on the bacterial community structure in each nanoparticles-amended soil.



(b)

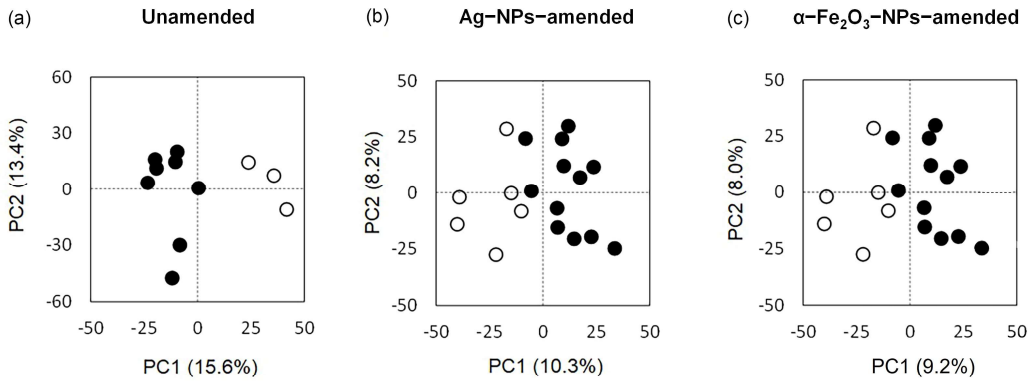
	F	R^2	*p*
Plant	1.317	0.086	**0.002**
Treatment	1.015	0.068	0.427
Plant × Treatment	1.111	0.217	0.779

Figure 5. (**a**) Principal component analysis (PCA) with all bacterial genera in the uncultivated soil amended with Ag-NPs (○), rhizosphere soil of the common bean plants amended with Ag-NPs (●), uncultivated soil amended with α-Fe_2O_3-NPs (□), rhizosphere soil amended with α-Fe_2O_3-NPs (■) and (**b**) perMANOVA test to determine the effect of plant cultivation, nanoparticles application and their interaction.

The PCA did not separate the communities considering all the putative metabolic functions in the rhizospheric and uncultivated soils amended with nanoparticles (Figures S7 and S8). The perMANOVA test showed that the cultivation of the common bean plants did not change the putative functional profile of the soil bacterial community.

3.7. Changes in the Bacterial Community over Time

The PCA with all the bacterial groups assigned up to the taxonomic level of genus clearly separated the bacterial community at the onset of the experiment from that after 100 days, independently, of the application of nanoparticles or the cultivation of the common bean plants (Figure 6). Time had a highly significant effect on the bacterial community structure.



(d)

	F	R^2	*p*
Unamended	1.582	0.149	**0.006**
Ag−NPs−amended	1.581	0.090	**0.001**
α−Fe_2O_3−NPs−amended	1.248	0.072	**0.008**
Treatment × Day	1.317	0.084	0.229

Figure 6. Principal component analysis (PCA) with all bacterial genera in the (**a**) unamended, (**b**) Ag-NPs-amended and (**c**) α-Fe_2O_3-NPs-amended soil at the onset of the experiment (○) and after 100 days (●); (**d**) permutational analysis of variance (perMANOVA) test used to determine the effect of time on the bacterial genera and the interaction between treatment and time (Day).

The PCAs did not separate the putative metabolic function structures at the onset of the experiment from those after 100 days. The perMANOVA test showed that the putative functional profile of the bacterial community in the soils did not change over time (Figure S9).

4. Discussion

4.1. Plant Characteristics

The application of nanoparticles to arable soils has often been studied [39–41]. For instance, Fe or Fe_2O_3-NPs have been applied to soil to reduce Fe deficiencies [8]. Iron is essential for the formation of chlorophyll in plants and its deficiency causes chlorosis [42]. Positive effects of the application of Fe-NPs on the morphological characteristics of some plants have been reported. For instance, Sutariya, Vyas, Faldu, et al. [43] found that the application of Fe-NPs at concentrations between 0.5 and 2 g kg^{-1} soil increased the root length and chlorophyll content of rice plants (*Oryza sativa* L.). In this study, however, the application of Ag-NPs or α-Fe_2O_3-NPs at 0.15 g kg^{-1} soil did not affect the morphological characteristics of the bean plants. These differences might be due to the type of plant cultivated, the concentration of the nanoparticles and/or the soil characteristics.

In this study, the application of Ag-NPs reduced the Cu, Fe, Zn and Mn content in the roots and Mn in the aerial part of the bean plants. These results are similar to those previously reported by Zuverza-Mena, Armendariz, Peralta-Videa, et al. [44], who determined that

Ag-NPs reduced the amount of Cu, Mn and Zn in radish sprouts (*Raphanus sativus* L.). A decrease in the heavy metal concentrations in plants could be due to the formation of NPs aggregates, which reduce water transport and affect nutrient acquisition pathways [45–47]. This can reduce the heavy metal content in the plant, as the roots play a key role in the absorption of metal ions from the soil [48].

De Souza, Govea-Alcaide, Masunaga, et al. [49] reported that the application of magnetite nanoparticles (Fe_3O_4-NPs) increased the accumulation of nutrients, such as, P, K, Ca, Mn and Fe, in the roots, stem and leaves of common bean plants. In this study, however, common bean plants cultivated in soil amended with α-Fe_2O_3-NPs had lower Mn, Zn and Fe content in the roots compared to plants cultivated in the unamended soil. In the aerial part of the plants, the content of heavy metals was not significantly different between the amended and unamended plants. Yang, Alidoust and Wang [50] reported that the foliar application of Fe_2O_3-NPs with a size of 5 nm increased the uptake of Fe in soybean (*Glycine max* L.) compared to plants when the nanoparticles were applied to the soil. Tombuloglu, Slimani, AlShammari, et al. [51] reported an increase in the amount of Fe in the roots of barley (*Hordeum vulgare* L.) cultivated in hydroponics amended with Fe_2O_3-NPs. Rui, Ma, Hao, et al. [8] reported that an application of Fe_2O_3-NPs increased the Fe content in peanut plants (*Arachis hypogaea* L.). Yuan, Chen, Li, et al. [52] found that the application of Fe-NPs can be positive at low concentrations, while at a high concentration, the Fe-NPs formed aggregates and blocked the transport of nutrients, e.g., Fe in the roots of chili plants (*Capsicum annuum* L.). Thus, the size and concentration of the nanoparticles and the way they are applied may affect their uptake and determine the potential benefit to plants.

4.2. Diversity and Structure of Soil Bacterial Community

Some previous studies have reported that the application of Ag-NPs affected the soil microbial diversity [53]. On the one hand, Zhang, Huang, Zhang, et al. [54] reported that the application of Ag-NPs at a concentration of 100 mg kg^{-1} significantly reduced the species richness in a soil cultivated with cucumber (*Cucumis sativus* L.), while it increased the species richness in uncultivated soil. On the other hand, Shah, Collins, Walker, et al. [55] reported that application of Ag-, Ni-, Fe- or Co-NPs did not reduce the species richness when they were applied on the soil surface. In our study, the nanoparticles did not change the alpha diversity of the bacterial community. These differences found when Ag-NPs were applied to soil might be due to the complexity of the soil–plant–microbiome system. Bacteria and plant exudates, as well as the physicochemical properties of soil can affect the mobility and the transformation of nanoparticles and modify their bioavailability and toxicity [10]. Furthermore, although the toxicity of NPs can affect some microbial groups, these can be replaced by resistant or less sensitive microorganisms, thus maintaining species diversity.

The dominant phyla in the bacterial community, i.e., Proteobacteria, Actinobacteria and Acidobacteria, as well as one of the most abundant genera, i.e., *Bacillus*, are widely distributed in ecosystems and often dominate in soil [6,56–58].

4.2.1. Effect of Nanoparticles Application on the Structure and Taxonomic Composition of the Soil Bacterial Community

There are a limited number of studies about the effect of Fe_2O_3- and Fe_3O_4-NPs on the structure of soil bacterial communities. He, Feng, Ren, et al. [7] reported that γ-Fe_2O_3-NPs changed the structure of the bacterial community, while Fe_3O_4-NPs had no such effect. Cao, Feng, Lin, et al. [59] found that Fe_3O_4-NPs altered the structure of the soil bacterial community in the rhizosphere of corn (*Zea mays* L.). Iron oxides are found naturally in soil, where they form nano-sized aggregates; therefore, it can be assumed that microorganisms are well-adapted to iron limiting a possible effect, as was found in this study [60,61].

Some previous studies reported that the application of Ag-NPs altered the soil bacterial community. Wang, Shu, Zhang, et al. [11] found that the application of Ag-NPs at a

concentration of 100 mg kg^{-1} soil changed the bacterial community structure after 7 days of exposure, while McGee, Storey, Clipson, et al. [62] reported that the application of Ag-NPs at 50 mg kg^{-1} soil altered the bacterial community structure in a pasture's soil. In this study, no change in the bacterial community structure was detected 100 days after the application of Ag-NPs to the soil. This indicates that the effect of the nanoparticles on the soil microbiome was related to multiple factors and their interaction, such as the time of exposure, the composition and physicochemical properties of the soil, and the presence and type of cultivated plant. The antimicrobial activity of metallic and metal-oxide nanoparticles is mainly attributed to direct contact with the bacterial cell wall. This interaction can alter the plasma membrane, generate reactive oxygen species and damage DNA [63,64]. The effect of nanoparticles on soil bacteria may be determined by the physical interaction between them. In a complex ecosystem such as soil, this interaction can be limited by various factors, such as the nanoparticles' concentration and distribution, the interaction between the nanoparticles and soil components or plant-derived compounds.

Although the changes in the bacterial community structure were not significant when the nanoparticles were applied to the soil, the relative abundance of some specific bacterial groups changed substantially. The application of Ag-NPs increased the relative abundance of *Rhizobium* and *Bradyrhizobium* in the non-rhizosphere soil compared to that in the unamended soil. Members of *Rhizobium* are nitrogen-fixing bacteria that are associated with the roots of plants, such as the common bean and chickpeas (*Cicer arietinum* L.) [65]. The increase in the relative abundance of nitrogen-fixing groups after the application of Ag-NPs has been reported before. Zhang, Huang, Zhang, et al. [54] reported that the relative abundance of *Bradyrhizobium* increased significantly in uncultivated soils and soils cultivated with cucumber plants (*Cucumis sativus* L.) amended with Ag-NPs. Similar results were reported by Shah, Collins, Walker, et al. [55] and Meier, Dodge, Samarajeewa, et al. [66], who detected an enrichment of *Bradyrhizobium* in soils amended with Ag-NPs. Wu, Zhai, Liu, et al. [67] reported an increase in the relative abundance of *Mesorhizobium* after the application of Ag-NPs. Previous studies have suggested that Gram-positive bacteria are more susceptible than Gram-negative bacteria to Ag-NPs [68,69]. This could explain a higher tolerance of *Rhizobium*, which is Gram-negative, to these nanoparticles and its enrichment in this study.

4.2.2. Effect of Cultivation of Common Bean Plants on the Structure and Taxonomic Composition of the Soil Bacterial Community

The cultivation of common bean plants did not significantly change the alpha diversity of the soil bacterial community. Ling, Wang and Kuzyakov [70] reported a decrease in the bacterial diversity in the rhizosphere of cultivated plants compared to the arable bulk soil, while the diversity in the rhizosphere and bulk soil in forests was similar. This means that a possible effect of the rhizosphere on the bacterial community also depends on environmental conditions, e.g., the soil properties.

The structure of the bacterial community was different in the rhizosphere compared to the uncultivated soil, regardless of the type of nanoparticle applied. Plants excrete root exudates, which include low and high molecular weight compounds, such as amino acids, sugars, organic acids and proteins. These compounds are an important source of organic material in the soil, as they can be easily metabolized by microorganisms [71]. Root exudates might affect the nutrient availability and the diversity of bacterial groups in the rhizosphere, which are related to nutrient acquisition, protection against pathogens and plant stress resistance [54,72–74].

Some previous studies have reported that the presence of plants can modify the effect of nanoparticles on soil bacterial communities. Xie, Guo, Zhang, et al. [75] reported that the bacterial community in the rhizosphere might respond differently to the application of nanoparticles than that in the bulk soil. Ge, Priester, Van De Werfhorst, et al. [76] found that the cultivation of soybean reduced the effect of ZnO nanoparticles on the soil bacterial community, but that of the CeO_2 nanoparticles increased it. The plant might alter the effect

of the nanoparticles, as the characteristics of the rhizosphere are different from that in the bulk soil. This might be due to the secretion of root exudates, whose plant-specific composition can change in response to stress or other factors, such as nutrient and water availability [73]. Exudates can also alter soil properties, such as the pH, enrich beneficial microorganisms and/or chelate contaminants [77]. Additionally, root exudates can alter the aggregation, dissolution and sedimentation of metal nanoparticles, and, consequently, their toxicity [78,79].

4.2.3. Effect of Time of Exposure

The time of exposure significantly changed the bacterial community structure, regardless of the application of nanoparticles and the cultivation of plants. This is similar to the results reported by Ge, Priester, Van De Werfhorst, et al. [76], who determined temporary changes in the bacterial community in soil with and without CeO_2- and ZnO-NPs and with and without soybean plants. These differences over time can be related to dying roots and root exudates that change the availability and composition of the soil C. These changes in the C substrate availability altered the bacterial community structure.

Long-term exposure may increase or limit the effect of nanoparticles on some bacterial groups. Wu, Zhai, Liu, et al. [67] found that a short-term 7-day exposure to Ag-NPs (50 mg kg^{-1} of soil) did not affect the diversity or structure of the bacterial community in the lettuce (*Lactuca sativa* L.) rhizosphere, but did alter it when the exposure was increased to 63 days. Contrarily, Zhai, Chen, Liu, et al. [80] reported that after a long-term incubation (60 days), the impact of TiO_2-NPs on the bacterial community decreased and allowed the bacteria to recover. On the one hand, the reduction in toxicity may be related to community resilience, i.e., the ability to recover after a disturbance caused by exposure to a pollutant [81]. On the other hand, the transformation and the interaction of NPs with the soil components take time, which could reduce their possible negative effect. For instance, Tian, Kah and Kariman [82] reported that nanoparticles can be retained on the clay fraction in a soil, which might reduce their toxicity.

4.3. Putative Functional Profile of the Bacterial Community

The application of nanoparticles, the cultivation of bean plants and time did not significantly change the putative functional profile of the bacterial community. However, the variation between the samples was lower in the functional profile than in the taxonomic bacterial profile, which could be related to a functional redundancy in the bacterial community. This means that more than one bacterial species had the capacity to perform the same metabolic function [83]. As such, although the application of nanoparticles affected specific bacterial genera, this was not mirrored at the functional level. Zhai, Chen, Liu, et al. [80] found that although the application of TiO_2 nanoparticles modified the composition of the bacterial community in the soil, these changes were not reflected at the functional level or in the enzymatic activity. It is therefore possible that different functional processes are maintained in an ecosystem, even when it undergoes a disturbance and the bacterial community structure is altered and not all bacteria recover from it [81]. It must be remembered, however, that the metabolic functions were predicted, and a shotgun metagenomics analysis might be required to confirm the results reported here.

5. Conclusions

The application of nanoparticles had a negative effect on the metal content, i.e., Zn, Fe, Mn and Cu, of common bean plants. The cultivation of beans and the time of exposure, i.e., temporal changes, had a larger effect on the bacterial community structure than the application of the nanoparticles to the soil. More in-depth studies, e.g., shotgun metagenomics, might be required to determine the mechanism by which the plant can affect the effect of nanoparticles on the different soil microorganisms. This highlights the importance of considering multiple factors and their interactions when assessing the environmental impact of nanoparticles in a complex system, such as soil.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13092341/s1, Figure S1: Soil sampling, experimental design and characterization; Figure S2: Effect of the application of nanoparticles on the alpha diversity of the soil bacterial amplicon sequences variants (ASVs); Figure S3: Effect of cultivation of bean (*Phaseolus vulgaris* L.) rhizosphere (R) versus uncultivated (UC) soil, on the alpha diversity of the soil bacterial amplicon sequence variants (ASVs); Figure S4: Volcano plot comparing the relative abundance of the bacterial groups assigned to the taxonomic level of genus in the amended non-rhizosphere soils versus the unamended non-rhizosphere soils and the amended rhizosphere soils versus the unamended rhizosphere soils; Figure S5: Principal component analysis (PCA) with all the putative metabolic functions in the three soils left unamended or amended with nanoparticles and perMANOVA test to determine the effect of application of the different nanoparticles on the putative metabolic functions at day 100; Figure S6: Volcano plot comparing the relative abundances of bacterial groups assigned to the taxonomic level of genus, comparing rhizosphere versus uncultivated soils amended with the nanoparticles; Figure S7: Principal component analysis (PCA) with all the putative metabolic functions in the rhizosphere and uncultivated soils amended with nanoparticles; Figure S8: Principal component analysis (PCA) with all the putative metabolic functions in the rhizosphere and uncultivated soils amended with nanoparticles and perMANOVA test to determine the effect of plant cultivation, nanoparticles application and their interaction; Figure S9: Principal component analysis (PCA) with all the putative metabolic functions in soils at the onset of the experiment and after 100 days, and perMANOVA test to determine the effect of time on the putative metabolic functions; Table S1: Methods used to determine soil characteristics; Table S2: Statistical analysis applied to the data and the package in R used; Table S3: Some morphological characteristics of common bean plants (*Phaseolus vulgaris* L.); Table S4: Heavy metal content in the roots and leaves of common bean plants (*Phaseolus vulgaris* L.); Table S5: Bacterial genera with a large effect size when soil was applied with Ag-NPs and α-Fe_2O_3-NPs; Table S6: *p* values obtained from permutation test of multivariate homogeneity of groups dispersions.

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