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Quantitative Determination Of Functional Bacteria Associated With Biogeochemical Cycles In Rhizospheric Soils Of Cofee Cultivation (Coffea Arabica)

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Abstract. The microbiota present in the soil is usually diverse and plays a vital role in the cycling and availability of nutrients for plants, being one of the potential indicators of soil quality. The objective of this work was to determine the population densities of the entire microbial groups and cultivable functional bacteria associated with the biogeochemical cycles of the soil of the coffee biosphere in the municipality of Lourdes, Norte de Santander. Three farms were sampled, and two rhizome soil samples were taken on each farm at a depth of 20 cm. Total microbial groups were counted on plates in Nutrient Agar (AN), Rose Bengal Agar (ARB), and Oat + Nystatin Agar (ANA) media. For the quantification of cellulose, starch, protein, chitin, and phosphorus solubilizing bacteria, selective media and plate count were used and, by the NMP method, a symbiotic bacteria that fix atmospheric nitrogen and nitrifying bacteria. In turn, correlations were determined between the density of the functional groups found and the specific physicochemical properties of the soils. The results showed statistically significant differences ($p>0.05$) in the population density variable in the total microbial groups. Similarly, statistically significant correlations ($p\leq0.05$; ≤0.01) were observed between the population density of some functional groups and the physicochemical properties of rhizosphere soils. The soil nutrients presented the most correlation in this work were Na, Ca, and P, followed by OM and K.

Keywords: microbial functional groups, Microbiota, soil microorganisms, bacterial count.

Introduction

Coffee is considered the main agricultural export product, around four million Colombians depend on this crop from planting to final consumption, and 25% of the rural population produces coffee (Federación Nacional de Cafeteros-FNC, 2014). More than 80 countries cultivate it, and more than 50 export it, reaching a worldwide export of 135.2 million 60-kg bags of coffee in the 2019/2020 period (United States Department of Agriculture-USDA, 2021). At the national level, it is constituted as one of the most economically important crops since around 22 departments produce it, represented in 853,698 ha cultivated as of 2019 with a production of 14.8 million bags of coffee, the highest in the last 25 years (Ministry of Agriculture and Rural Development-MADR, 2020). In addition, the department of Norte de Santander has a share in production represented by 36 coffee municipalities with approximately 23,027.00 hectares of planted area (MADR, 2020; Information and Communication Network of the Agricultural

Sector-AGRONET, 2019).

According to the Departmental Committee of Coffee Growers of Norte de Santander, 90% of the coffee planted prevails as an agroforestry system in conjunction with species such as banana, fruit, and timber that provide shade and allow the farmer other sources of income (Rodríguez and Mora, 2014). Agroforestry systems favor the biodiversity of the crop, the presence of natural enemies, the recycling of nutrients, and the increase of ecosystem services (Cerda et al., 2020). However, producing coffee in full sun and under conventional management implies higher expense management and greater demand for chemical fertilizers and pesticides that negatively affect the well-being of microorganisms and their biochemical activity (Paolini, 2018).

The rhizospheric soil is influenced by the metabolism of plants that secrete several carbon-rich radical exudates that can favor the presence of microbiomes (Chen et al., 2016), indicators of soil quality and health. Therefore, it is susceptible to changes caused by agricultural management practices, environmental changes, and organic matter dynamics (Du et al., 2021). One of the functions that these microbiomes fulfill in the soil is the mineralization of organic matter to forms that are more assimilable by plants. Therefore, these microbial functional groups participate in a specific stage of the transformation of organic and inorganic compounds. of C, P, N, and S and are directly involved with the stages of biogeochemical cycles causing significant changes in the chemical properties of soils by recycling nutrients and improving the availability of nutrients for plants (Kour et al., 2020). The population is grouped in biogeochemical cycles, atmospheric nitrogen-fixing and nitrifying bacteria, phosphorus solubilizers, amylolytic, cellulolytic, proteolytic, chitinolytic, and pectinolytic microorganisms, among others (Ramírez et al., 2013).

In recent years, agricultural practices such as the indiscriminate use of chemical inputs (pesticides and fertilizers), monoculture, and intensive tillage have altered the organic composition of the soil, the ecological balance, and the metabolic function of the edaphic microbiota (Hernández et al., 2013). Therefore, the study of the population of microbial functional groups in soils allows knowing the potential of this biological resource in crops of regional economic interest. Therefore, the objective of this study was to quantify the population density of the functional groups related to the Carbon, Nitrogen, and Phosphorus cycle in rhizospheric soils. Also, the determination of the effect of some physical and chemical properties of the coffee-growing soil on the population of these functional groups serves as base information to generate conservation strategies to bioincrease the population and biological activity in situ and to use them as biological inoculants that improve the rhizosphere effect, nutrient cycling, phytoprotection, and crop productivity is analyzed.

Materials and Methods

Sampling and physicochemical analysis.

The sampling was carried out on three farms in the municipality of Lourdes, Norte de Santander. In each lot, 5 subsamples of rhizosphere soil were taken, and a composite sample of approximately 1 kilogram was formed using zig zig transects (Torres and Lizarazo, 2006). These areas were georeferenced using the

Global Positioning System (GPS) (Table 1). The samples were stored in hermetic bags and at 4°C. They were taken to the Bioprocess Laboratory of the Francisco de Paula Santander University (UFPS), and the microbiological analysis was performed within 48 hours of taking the sample. For the physicochemical analyses, they were sent to the UFPS soil laboratory, and the Hydrogen potential (pH), texture, electrical conductivity, and the content of Nitrogen (N), Phosphorus (P), Carbon (C), Calcium (Ca), Magnesium (Mg), Potassium (K) and % Organic Matter (OM).

Table 1. Location of sampling areas

Estate	Sidewalk	Location	Geographic
La Rinconada	El Alto	07°55'47.6' N 072°49'50.8' W	1662 m.s.n.m.
El oriente	Volcanes	07°55'23.4' N 072°50.07.8' W	1780 m.s.n.m.
El llano	Volcanes	07°56'13.1' N 072°50'.04.3' W	1480 m.s.n.m.

Preparation of serial dilutions.
Serial dilutions were made up to 10-6 from each rhizospheric soil sample. Each functional group took a volume of 100 µL from each dilution and sown on the surface in the differential culture media.

Count of total bacteria, fungi, and actinomycetes.
Dilutions from 10-2 to 10-6 were inoculated in Nutrient Agar (AN) for bacteria, Rose Bengal Agar for fungi (ARB), and in Oat + Nystatin Agar (ANA) for actinomycetes. The boxes were incubated at 28°C for 48 hours for AN, 3-5 days for (ARB), and 8 days for (AAN). It was quantified using the plate count technique (CFU/mL) in triplicate (Ramírez-Elías et al., 2014).

Count of functional bacteria of the nitrogen cycle.
For the count of nitrogen fixers, 100 µL of the 10-2 to 10-6 dilution were inoculated into three vials with semi-solid media NFb, JNFb, JMV, and LGI-P. The inoculated vials were incubated for 7 days at 30-32°C until the formation of a subsurface film as an indicator of positive growth and/or color change of the medium. The count was performed using the Most Probable Number method, applying the McCrady table (Dobereiner et al., 1995). Actinomycetes were counted from 10-2 to 10-6 dilutions in Ashby agar in triplicate, and the dishes were incubated at 28°C for 8 days. The NMP method was used for five tubes with ammonium broth to count nitrifying bacteria. From the 10-1 to 10-6 dilution, 100 µL were inoculated in each vial and incubated at 28°C for three weeks. The Griess-Ilosvay reagent was added to each vial to detect nitrites, considering vials that turned pink to red to be positive (Montaño et al., 2013).

Count of functional groups of bacteria of the phosphorus cycle.

From the dilutions of 10-2 to 10-6, 100 µL were seeded in triplicate in the SRSM medium using tricalcium phosphate as the phosphorus source. The boxes were incubated at 28°C for 3-5 days (Avellaneda and Torres, 2015). The positive colonies were those that presented a degradation halo.

Counting functional groups of carbon cycle bacteria.
We started with dilutions of 10-2 to 10-6, and 100 µL were inoculated by surface and triplicate in the selective media. The dishes will be incubated at 28°C for 3-5 days for cellulolytic and amylolytic bacteria and 5-8 days for chitinolytic bacteria. For cellulolytic microorganisms, the culture medium described by Tangapo et al. (2018) with 1% carboxymethylcellulose as the sole carbon source. The positive colonies formed surrounding degradation halos, revealed with the addition of Congo red at 1M.

For chitinolytic microorganisms, the chitin medium described by Castro et al. (2011) using chitin as the only carbon source obtained from shrimp extract. Positive colonies were those that could grow in the medium. For the quantification of amylolytic, the culture medium described by Tangapo et al. (2018) with 10% starch as the only carbon source. The positive colonies formed surrounding degradation halos, revealed with the addition of Lugol.

Analysis and interpretation of results.
Due to non-compliance with the statistical assumptions of normality and homoscedasticity of the variances, all the original counts for each microbial and functional group were analyzed using the Kruskal-Wallis non-parametric analysis of variance. When the probability value $p<0.05$ was observed, the Kruskal-Wallis rank test was used to compare means. Pearson's correlation analysis (r) was performed to determine the degree of relationship between the population counts of the microbial groups and the physicochemical properties of the soil. The execution of all the analyzes was handled with INFOSTAT version 1.1 under Windows (Infostat, 2002).

Results and discussion

Physicochemical analysis of rhizosphere soils. The soils were characterized as medium to finely textured clay loams, this type of soil may be appropriate for cultivation since it allows moderate water permeability. Table 2 shows that the OM content was between medium and high, with values between 3.89% and 5.31%, as well as the % of N and % of C. On the other hand, the pH was very low in the three farms, characterized as strongly acidic soils, and not highly recommended for cultivation since the pH range of 5.0 to 5.5 is considered adequate (FNC, 2016). However, there were medium and high contents of P and Ca in a range of 21 to 156.5 ppm and 3.1 to 12.92 milliequivalents/100 gr of soil. In all the farms, medium levels of Mg and low levels of Na were presented. Soluble K content in soils is low because it leaches in acid soils.

Table 2. Average result of the physicochemical analyzes of the rhizospheric soil samples of the sampled farms.

Physicochemical parameter	La rinconada	El Oriente	El Llano
pH	4,25	4,5	4,5
% MO	3,89	5,31	4,1
% C	2,25	3,07	2,39
% N	0,18	0,24	0,20
K	0,25	0,25	0,2
P	156,5	21	86,75
Ca	12,92	6,31	3,1
Na	0,095	0,07	< 0,1
Mg	2,14	1,21	0,97
Al	2,18	2,01	2,28

Colombian soils are strongly acidic or moderately acidic, which is why one of the traditional agricultural practices is liming the soil. In the case of coffee cultivation, acidity is one of the most notorious problems, finding that 63% of the samples analyzed from Norte de Santander have a pH < 5 (FNC, 2016). The organic matter is due to the presence in the soil of plant residues, macro and microfauna, and/or the addition of animal droppings such as manure and chicken manure (Song et al., 2016; Yanardag et al., 2017). In addition, organic matter contributes to N and P reservoirs and is another nutrient source used by the soil microbiota with specific physiological activities such as cellulose degraders, pectin, and phosphorous compounds (Ramírez-Elías et al., 2014).

Concerning the content of Nitrogen in natural and agricultural soils, they are low, which is why it demands the application of fertilizers such as urea or the contribution of organic matter so that through the mineralization of this, the required concentrations of Nitrogen can be obtained (Cerón and Aristizabal, 2012). The availability of soluble phosphorus in the soil is low because it is very reactive and reacts rapidly with calcium cations in alkaline soils and aluminum and iron in acid soils (Kour et al., 2021). Therefore, the presence of high levels of P in the soils studied may be due to the application of high phosphorous fertilizers in agricultural soils to increase the easily usable phosphorus by plants. Therefore, it is required to increase its concentration by applying fertilizers (Meena et al., 2014). Another essential macroelement is Ca, and its availability in the soil can be affected by acid pH; when the concentrations are low in the soil, the young tissues of the plant are affected because they are not very mobile in it (Díaz et al., 2007). The low concentrations of Mg in the three farms are related to the strongly acidic soils. In studies carried out, they have found that the equilibrium relationships of Ca, Mg, and K are essential for good assimilation of nutrients since there may be an antagonist between them in their availability in the soil and absorption by the plant (Díaz et al., 2007).

Population density of the total microbial groups in the rhizosphere soil of the coffee crop. Figure 1 shows the microbial population density in coffee cultivation. Significant statistical differences ($p<0.05$) were found in each quantified microbial group. It was observed that the "La Rinconada" and "El Llano" farms

showed statistical differences ($p<0.05$) in the total fungi and actinomycetes microbial groups concerning the "El Oriente" farm, which reported the lowest density, only the farm "El Llano" was different in the population density of total bacteria (1.73E+05 CFU/g of soil). A total fungal population density of 4.62E+04 CFU/g of soil and a total actinomycetes density of 5.83E+04 CFU/g of soil were found on the "La Rinconada" farm (figure 1). Bacterial populations of 2.62 and 3.69 Log CFU/g of soil, respectively, have been reported in the rhizosphere of potato crops, and for fungal populations, the counts ranged between 4.45 and 4.83 Log CFU/g. (Beltran, 2014). Also, Zhang et al., 2012 reported that in the maize rhizosphere, the average populations of bacteria, fungi, and actinomycetes were 7.3E+06, 1.4E+04, 1.9E+09 CFU/g of soil, respectively. The above shows a microbiological variability in the populations of bacteria, fungi, and actinomycetes in the rhizosphere of the agricultural plants and the sampled farms, possibly due to the edaphological, landscape, climatic, and crop management characteristics, among others. Also, cropping systems can have differential biogeochemical properties in the short and medium term. Such properties as Redox reactions and soil structure can affect microbial life in the soil (Vanegasa et al., 2013). In addition, within the microbial populations, there are different microorganisms associated with the rhizosphere that have been reported as helping to promote plant growth and increase crop productivity, thanks to different mechanisms such as the synthesis of phytohormones, the dissolution of minerals, and the production of siderophores (Alí and Glick, 2019).

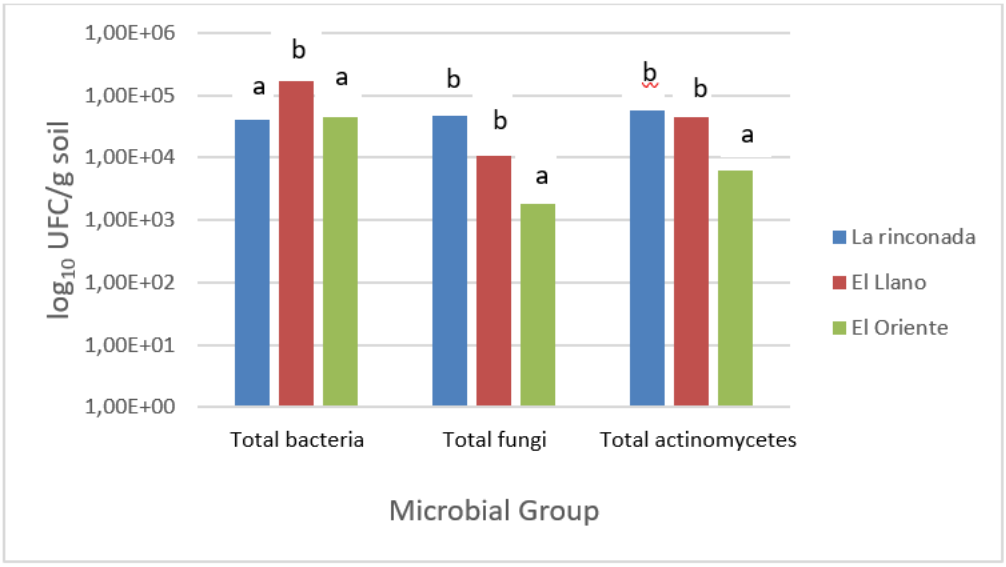

Figure 1. Count of total microbial groups in coffee farms.
Different letters indicate statistical differences ($p<0.05$) (n=6)

Functional microbial groups in the carbon cycle (C) in the rhizosphere soil of coffee cultivation.
Cellulolytic, amylolytic, and chitinolytic bacteria are functional groups of microorganisms that are important in decomposing soil organic matter. Statistical differences ($p<0.05$) were observed in the cellulolytic and chitinolytic functional groups when counting bacterial populations. However, no significant differences ($p>0.05$) were found in the Amylolytic functional groups (Table 3). The coffee rhizosphere soil of the "La Rinconada" farm was the one that showed the highest average population of the Cellulolytic

functional group. In general, the average population densities of bacteria in the coffee farms were observed, showing a relatively uniform behavior, probably due to the similar physicochemical characteristics of the soils of the sampled production units. This functional group of bacteria has a significant role in carbon mineralization and stabilization processes related to the intrinsic resistance to the degradation of organic compounds (Yanardağ et al., 2017).

Table 3. Count microbial groups related to the carbon cycle and proteolytic bacteria in coffee rhizosphere soils.

	Functional Groups of Bacteria (UFC/g SOIL)		
Estate	**Cellulolytic**	**Amylolytic**	**Chitinolytic**
El Llano	3,83E+03 a	9,83E+03*	8,50E+04 b
El Oriente	4,67E+03 a	4,50E+03	1,67E+04 a
La Rinconada	2,97E+04 b	1,60E+04	4,85E+04 ab

Different letters indicate statistical differences ($p<0,05$)
*: No statistically significant differences were found ($p>0,05$). (n=6)

In general, the population biodiversity of the functional groups related to the carbon cycle showed variability depending on the crop and farm studied. However, it is almost a rule that the population density of bacteria has a close relationship with some physical and chemical properties of the soil, as well as other factors that negatively affect the populations, such as the application of agrochemicals, discharge of pollutants into the soil and the presence of antagonistic organisms (Beltrán and Lizarazo, 2013).

Functional microbial groups related to the Nitrogen cycle in the rhizosphere soil of coffee cultivation.

The average counts of nitrogen-fixing actinomycetes and nitrifying bacteria are shown below. Table 4 shows that a statistical difference ($p<0.05$) was found in the average number of cells in the Nitrifying bacteria group but not in the functional group of diazotrophic Actinomycetes ($p>0.05$). Various studies have shown that the structure and diversity of microbial populations in plant production systems are affected by the application of chemical fertilizers, organic amendments, pesticides, the introduction of biofertilizers, and crop water management (Vanegasa et al., 2013; Zhao et al. al 2016; Yanardağ et al. 2017). Finally, a process of great importance for the entry of nitrogen into the soil-plant system is

nitrification, and this is carried out when nitrifying bacteria in the presence of oxygen oxidize NH4+ into NO3-, which has as an intermediate process the production of NO2-; That is why the production of NO3- is significant in agriculture since it is very mobile in the soil and easily assimilated by plants (Treseder, 2008).

Table 4. Count functional microbial groups: nitrogen-fixing actinomycetes and nitrifying bacteria from rhizosphere soils of coffee cultivation.

Estate	Diazotrophic functional groups	
	Actinomycetes	**Nitrifying bacteria**
	UFC/g Soil	Cel./g Soil
El Llano	4,00E+04 *	6,58E+01 a
El Oriente	2,78E+04	2,01E+02 ab
La Rinconada	5,65E+04	4,77E+02 b

Different letters indicate statistical differences (p<0,05)
*: No statistically significant differences were found (p>0,05)

Concerning Nitrogen-fixing bacteria, the largest population of diazotrophic bacteria was found in the El Llano farm, predominating the populations in the selective media LGI-P, JNFB, and JMV for Gluconacetobacter, Herbaspirillum, and Burkholderia (Table 5). The presence in general in the three crops studied may be due to the acidic pH requirements of these genera (Arguello and Moreno, 2014).

Table 5. Count nitrogen-fixing bacteria (NFB) in various selective culture media of coffee rhizosphere soils.

Estate	Selective culture media (Cel./g Soil)			
	LGI-P	**JNFB**	**NFB**	**JMV**
El Llano	8,83E+04*	4,48E+04 b	2,43E+04 b	8,23E+03 b
El Oriente	5,50E+04	1,50E+02 a	1,28E+04 ab	3,83E+04 b
La Rinconada	4,73E+04	1,33E+02 a	4,48E+03 a	4,33E+02 a

LGI-P, JNFB, NFB, and JMV: Selective media for counting Nitrogen-fixing bacteria

Different letters indicate statistical differences (p<0,05)
*: No statistically significant differences were found (p>0,05)

Functional microbial group: phosphorus-solubilizing bacteria related to the phosphorus cycle in the rhizospheric soil of coffee cultivation.

Figure 2 shows the population density of this functional group, with no significant statistical differences (p>0.05). Becerra et al. (2011) found similar counts in Uchuva cultivation, considering that soils with acidic pH limit their growth because only acid phosphatase enzymes can act. The addition of organic matter influences the increase in phosphatase activity and microbial phosphorus content, which contributes to the increase in available P. Therefore, the % of OM may be related to P's population of bacteria solubilizers (Sakurai et al., 2008). Soils rich in organic matter present a higher activity of alkaline phosphatase and increase the diversity of genes that encode this activity in the microbial communities of agricultural soils, which suggests that the activity and biomass of microorganisms play a fundamental role in the phosphorus mineralization, mainly in the rhizosphere (Alori et al., 2017). Hence, microorganisms' diversity and population density are indicators of interest in studying the quality and sustainability of terrestrial ecosystems (Reyes and Valery, 2007).

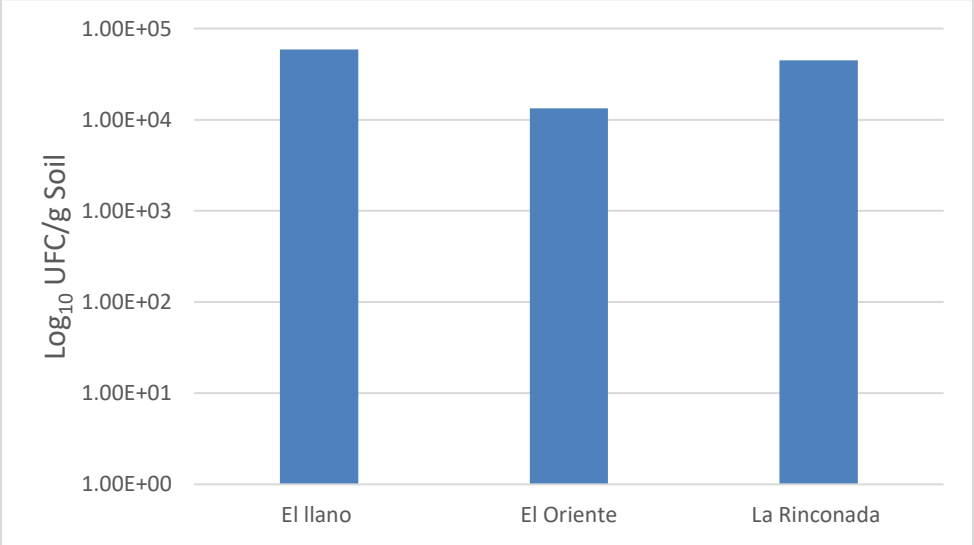

Figure 2. Count of bacteria associated with the P cycle in coffee farms. Different letters indicate statistical differences (p<0.05) (n=6)

Relationship of the chemical characteristics of the rhizosphere soil and the microbial and functional groups of the Carbon, Nitrogen, and Phosphorus cycles. Table 6 shows the Pearson correlation matrix of the population density variables of the microorganisms belonging to the microbial group and the related functional groups, and the edaphological characteristics of the rhizosphere soils.

Table 6. Pearson correlation matrix of microbial and functional groups versus chemical characteristics of rhizosphere soils.

	BT	HT	AT	BSP	BCL	BAL	BQL	BPL	AFN	BNF
pH	0,52	-0,98	-0,70	-0,62	**-1,00***	-0,89	0,04	-0,95	-0,91	-0,95
MO	-0,35	-0,75	-0,99	**-1,00****	-0,59	-0,91	-0,76	-0,33	-0,89	-0,32
C	0,33	-0,77	-0,99	**-1,00***	-0,61	-0,92	-0,75	-0,35	-0,90	-0,35
N	-0,16	-0,87	-0,99	-0,98	-0,74	-0,97	-0,62	-0,51	-0,96	-0,50
P	**-1,00***	0,33	-0,27	-0,37	0,52	0,04	-0,88	0,75	0,087	0,75
K	-0,40	0,95	0,96	0,92	0,86	**1,0***	0,45	0,67	**0,99***	0,67
Ca	-0,77	0,87	0,43	0,33	0,96	0,69	-0,36	**1,00***	0,72	**1,00***
Na	0,30	0,78	**0,99***	**0,99***	0,63	0,93	0,73	0,38	0,91	0,37
Mg	-0,67	0,93	0,55	0,45	0,99	0,78	-0,23	0,99	0,81	0,99
ACl	0,77	0,33	0,81	0,87	0,12	0,59	0,98	-0,17	0,55	-0,18

Microbial group (CFU/g Soil): BT: Total Bacteria HT: Total Fungi AT: Total Actinomycetes BSP: Phosphorus Solubilizing Bacteria BCL: Cellulolytic Bacteria BAL: Amylolytic Bacteria BQL: Chitinolytic Bacteria BPL: Proteolytic Bacteria AFN: Nitrogen-Fixing Actinomycetes BNF: Nitrogen-Fixing Bacteria pH: hydrogen potential; MO: organic material; C: Carbon; N: Nitrogen ; P: Phosphorus; K: Potassium, Ca: Calcium; Na: Sodium; Mg: Magnesium; CIA: exchangeable acidity. * Indicates a significant difference in the correlation ($p \leq 0,05$).

10 high correlations with statistical significance were found ($p \leq 0.05$, ≤ 0.01). For example, the microbial group total actinomycetes (AT) and phosphorus solubilizing bacteria (BSP) showed a positive correlation with Na (r=0.99); amylolytic and proteolytic bacteria showed a positive correlation with K (r=1.0) and Ca (r=1.0). Likewise, the Nitrogen-fixing actinomycetes (AFN) microbial group presented a correlation with K (r= 0.99) and the nitrifying bacteria with Ca (r= 0.99). On the other hand, the group of total bacteria (BT) with P showed a negative correlation (r= -1.0), the Phosphorus solubilizing bacteria (BSP) showed a negative correlation with MO and C (r= -1, 0), and cellulolytic bacteria with pH (r= -1.0). The soil nutrients that showed the most correlation in this work were K and Ca, followed by P, MO, and Na.
Among the chemical properties that favor the activity of bacteria is a pH close to neutrality, low acidity, high content of organic matter, and high availability of some elements necessary for their metabolism, such as N, Ca, and Mg (Beltrán and Lizazo, 2013). In this study, K and Ca were the elements that showed correlation with various microbial groups.

Conclusions

The conditions and agronomic characteristics were similar among the crops studied, demonstrating that acid soils are one of the drawbacks that occur in coffee soils.
The microbial populations involved in the C, N, and P cycles are present in all three coffee crops studied despite some physicochemical properties of the soils that are not highly recommended.
A higher population density of total bacteria was reported in the three crops, reiterating that the bacterial population constitutes one of the main microbiomes in agroecosystems.
Soil nutrients that showed correlations with some soil microbial and functional groups were pH, organic matter, Carbon, Phosphorus, Potassium, Calcium, and Sodium.

Conflicts of interest: The author declares that no conflict of interest could jeopardize the validity of the results presented.

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