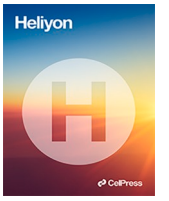
Heliyon 10 (2024) e28453

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Diversity and functional assessment of indigenous culturable bacteria inhabiting fine-flavor cacao rhizosphere: Uncovering antagonistic potential against *Moniliophthora roreri*

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A R T I C L E I N F O

Keywords:
Bacteria
Diversity
Cacao
Rhizosphere
Biocontrol
Moniliasis

A B S T R A C T

The Peruvian Amazonian native cacao faces ongoing challenges that significantly undermine its productivity. Among them, frosty pod rot disease and cadmium accumulation result in losses that need for effective and environmentally safe strategies, such as those based on bacteria. To explore the biological resources in the cacao soil, a descriptive study was conducted to assess the diversity of culturable bacteria across three production districts in the Amazonas region: La Peca, Imaza, and Cajaruro. The study also focused on the functional properties of these bacteria, particularly those related to the major issues limiting cacao cultivation. For this purpose, 90 native bacterial isolates were obtained from the cacao rhizosphere. According to diversity analysis, the community was composed of 19 bacterial genera, with a dominance of the Bacillaceae family and variable distribution among the districts. This variability was statistically supported by the PCoA plots and is related to the pH of the soil environment. The functional assessment revealed that 56.8% of the isolates showed an antagonism index greater than 75% after 7 days of confrontation. After 15 days of confrontation with *Moniliophthora roreri,* 68.2% of the bacterial population demonstrated this attribute. This capability was primarily exhibited by *Bacillus* strains. On the other hand, only 4.5% were capable of removing cadmium, highlighting the biocontrol potential of the bacterial community. In addition, some isolates produced siderophores (13.63%), solubilized phosphate (20.45%), and solubilized zinc (4.5%). Interestingly, these traits showed an uneven distribution, which correlated with the divergence found by the beta diversity.

Our results revealed a diverse bacterial community inhabiting the Amazonian cacao rhizosphere, showcasing crucial functional properties related to the biocontrol of *M. roreri*. The information generated serves as a significant resource for the development of further biotechnological tools that can be applied to native Amazonian cacao.

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https://doi.org/10.1016/j.heliyon.2024.e28453
Received 15 February 2023; Received in revised form 18 March 2024; Accepted 19 March 2024
Available online 21 March 2024

1. Introduction

"The food of the Gods" is the etymological meaning of *Theobroma*, the genus coined by the Swedish naturalist Linnaeus to the cacao tree, likely in reference to the divine and spiritual origin attributed by the Mesoamerican civilizations. This crop has invaluable cultural, political, and economic importance in the American and Mesoamerican territories, where ancient people have been using it because of its dietary and medicinal attributes [1]. *Theobroma cacao*, the source of chocolate, is native to the tropical region of South America and can be distinguished into eleven genetic clusters [2–4]. Among them, those classified into "fine flavor groups" have become increasingly popular, due to their exceptional quality, serving as primary raw material for the manufacture of high-end chocolates.

Peru occupies a prominent position as a producer and supplier of fine-flavor cacao, ranking as the world's second-largest producer of organic cocoa. It is expected to sustain its current upward trend in productivity (Ministry of Agrarian Development and Irrigation of Peru, 2022). Three native cacao groups have been recognized in Peru: "Chuncho", "Blanco Piurano'' and "Cacao Amazonas Peru''. The latter is highly cultivated in the Amazonas region and holds a protected designation of origin due to its unique organoleptic properties (INDECOPI, 2016) [5–7]. Moreover, it is an economically and socially relevant crop, serving as the major source of income for at least 90,000 Amazonian families (Ministry of Agrarian Development and Irrigation of Perú, 2019). However, this crop faces several challenges that concern smallholder farmers. Among them, a major issue is caused by *Moniliophthora roreri,* the causal agent of frosty pod rot disease (FPR) [8]. It infects cacao pods at all stages of development, and symptoms may appear 30 days after initial infection, including bean rotting and pod mummification with massive spore production on the surface [9,10]. According to Phillips-Mora and Wilkinson (2007), FPR has been more destructive and difficult to control than other major cacao diseases, such as black pod rot and witches' broom. Since its first appearance in Peru, it has become the most prevalent, severe, and serious in terms of yield losses [7,11,12].

Beyond diseases, another significant challenge is the susceptibility of cacao to Cadmium (Cd^{2+}) accumulation. Cadmium, a toxic heavy metal, can be naturally present in the soil or result from anthropogenic activities. It is introduced into the food chain through plant uptake, and its concentration depends on its bioavailability in the soil [13,14]. Due to the risks of cadmium to human health, regulatory agencies have adopted several actions to protect consumers from cadmium-contaminated products (European Food Safety Authority Commission Regulation, 2011; MERCOSUR, 2011; USDA, 2018). These include the setting of threshold levels, which when overpassed, cocoa bean parcels cannot be exported. In the Peruvian cacao regions of Piura, Tumbes, and Huanuco, samples have revealed bean values exceeding 0.96 μg/g [15,16], thereby overpassing the maximum limit of 0.80 μg/g established by Commission Regulation (EU) No. 488/2014. Similarly, in the Amazonas region, samples of root, leaf, testa, and cotyledon revealed high cadmium values in 59% of the evaluated plots [17]. Furthermore, recent findings indicate that 39% of the Amazonas territory exceeds the tolerable cadmium limit for agricultural land ($\geq$1.4 mg/kg) [18].

While not exclusive, these issues are of particular concern to smallholder farmers as they directly compromise the productivity and trade of their crops. Moreover, given the demand for organic food, there is a growing need for the development of sustainable agricultural strategies.

Fortunately, the plant has found strategic partners in the microorganisms, establishing complex and coordinated evolutionary relationships. These partnerships provide the basis of innovative biotechnological tools that complement conventional crop management. The microorganisms readily colonize most plant compartments, which provide specific biotic and abiotic conditions for microbial life [19]. Among the plant microenvironments, the rhizosphere, a narrow zone of soil surrounding and influenced by the plant roots, hosts an astounding number of microbes, which in response to the root exudates and low molecular weight compounds are recruited for colonization [20–22]. Among the rhizosphere microbiota, one particular group is receiving attention because of its beneficial effects on plants. The plant growth-promoting rhizobacteria (PGPR) represent a diverse category of bacteria that, through several mechanisms, exert positive effects on growth, organ development, immune responses, and hormonal signaling in plants. Moreover, they ensure the availability of essential nutrients, enhance their use efficiency, protect the host, and are environmentally safe. Bacteria associated with *T. cacao* have shown promising results as biocontrol agents for plant pathogens or as agents for immobilizing Cd. Some studies involving *Bacillus* strains have evidenced a significant reduction in the growth of *M. roreri*, *M. perniciosa*, or *Phytophthora* [23,24]. Similarly, the *Enterobacter* CdDB41 and *Klebsiella variicola* 18-4B strains showed substantial Cd immobilization rates under *in vitro* conditions [25,26]. However, despite these promising results, research in this area remains still scarce, especially concerning fine flavor cacao. Therefore, further research is required.

Historically, PGPR has been individually assessed. However, as previously mentioned, they belong to a complex bacterial community that cannot be ignored. Thus, a comprehensive and holistic approach considering the diversity of microbial species in the rhizosphere is necessary for a deeper understanding of the synergistic and cooperative mechanisms contributing to plant growth promotion and disease suppression. The diversity and abundance of bacteria can be explored through the direct recovery of the 16S rRNA gene. However, when it comes to assessing PGPR, direct isolation and cultivation strategies remain crucial. These strategies enable the measurement and manipulation of the functional, metabolic, and genetic properties of isolated strains, facilitating their further biotechnological approach.

In this regard, the focus of this work was to describe the diversity and composition of the rhizosphere culturable bacterial community associated with fine flavor cacao trees from representative cacao producer districts in the Amazonas region, Peru. Then, for functional assessment, to evaluate its potential to assess major cacao production issues, antagonistic assays, against *M. roreri*, and cadmium removal tests were conducted.

2. Material and methods

2.1. Sample collection

Rhizospheric soil samples were collected from native cacao trees cultivated under organic management systems in the Cajaruro (5°43′43.98″S; 78°20′59.06″W), Imaza (5°10′31.23″S; 78°17′10.57″W) and La Peca (5°37′44.80″S; 78°25′07.58″W) districts of the Amazonas region in Peru. Two collection points, each with three plants, were established for each district. The samples were taken at a depth of 10 cm, and stored in 50 mL sterile tubes at 4 °C until processing.

2.2. Bacterial isolation and culture conditions

Serial dilutions were performed using 1g of rhizospheric soil mixed with 9 mL of sterile 10 mM $MgCl_2$ solution. Aliquots of 100 μL were plated on a non-selective medium Tryptone-Yeast (TY) agar [27], supplemented with nystatin 5 ng/mL, and incubated at 28 °C for 24 h. Single colonies were selected and subcultured on TY medium under the aforementioned conditions. The selected isolates were grown in liquid TY medium and preserved with glycerol 30% (v/v) at −80 °C.

2.3. Bacterial identification

DNA extraction and purification were carried out using the Wizard®DNA extraction Kit (Promega, WI, USA) following the manufacturer's instructions. The 16S rRNA gene amplification was performed using universal primers 27 F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492 R (5′-TACGGYTACCTTGTTACGACTT-3′) [28]. The resulting 16S rRNA amplicons were sequenced (at Macrogen, South Korea) and aligned with reference type sequences, obtained from the National Center for Biotechnology Information (NCBI), Genbank Database (https://www.ncbi.nlm.nih.gov/), and The ribosomal database project (RDP) (http://rdp.cme.msu.edu/) using *MAFFT v7.490* package [29]. The phylogenetic tree was built with the *IQ-TREE* package [30]. Distances were calculated using a complete-deletion procedure. Phylogenies were constructed using the maximum-likelihood method, and the robustness of tree topologies was evaluated by bootstrap analysis (1000 replicates). To enhance the comprehension and visualization of diversity between and within genera, the Interactive *Tree Of Life* web-based tool (http://itol.embl.de) was used to display the clustering of each isolates on the phylogenetic tree [31]. The 16S rRNA sequences were deposited in the GenBank database, and the accession numbers are indicated in (Table 2).

2.4. Antagonism test against Moniliophthora roreri

Bacteria were cultured in liquid TY medium on a rotary shaker at 28 °C for 24 h up to the exponential growth phase ($OD_{600} = 0.5$). Next, a 100 μL aliquot of bacterial culture was spread onto Potato Dextrose Agar (PDA) plates. Then, a plug (7 mm) of growing mycelia of *M. roreri*, was placed in the center of the plate. Control plates were prepared, including non-bacterial inoculated + *M. roreri* and single bacterial PDA plates. The antagonism capacity index (AI) was measured at 7 and 15 days of incubation, following the modified formula: AI(%) = [1-(Da - 0.7)/(Db - 0.7)]x100; where Da is the diameter of *M. roreri* under bacterial confrontation, and Db is the diameter of the control [32].

2.5. Tolerance and cadmium removal capacity

Bacterial strains were cultivated in TY agar medium at 28 °C for 24 h. Single colonies were streaked onto TY medium supplemented with 0, 100 and 200 ppm of $CdCl_2$ using the replica plating method [33], and incubated at 28 °C for 72 h. For comparison, TY medium without any supplementation served as the control. Isolates displaying visible growth at both control and problem conditions were chosen and subjected to further testing using the drop-plate method. This step aimed to confirm their tolerance to cadmium. The selected bacterial strains were plated again under the same conditions described above. The tolerant bacterial strains were subsequently cultured in a liquid TY medium supplemented with 0, 100, and 200 ppm of $CdCl_2$ for 24 h at 28 °C. After incubation, the supernatant was obtained by centrifugation at 12 000 rpm for 6 min and filtered through a sterile filter with a pore size of 0.22 μm. The filtered solution was used for cadmium determination by atomic emission photometry (Agilent Technologies 4100 MP-AES). This analysis was performed by the Soil and Water Research Laboratory services (UNTRM, Peru). The removal cadmium efficiency (RE) was calculated as follows: $RE(\%) = ((C_0 - C_f/C_0) \times 100)$, where C_0 is the initial cadmium concentration in the medium and C_f is the cadmium concentration in the filtered solution [34].

2.6. PGPR-traits characterization

To determine siderophore production, aliquots of 10 μL bacterial strain, previously cultured as described in section 2.4, were inoculated in TY agar medium and incubated at 28 °C for 24 h. Then a layer of Cromo Azurol S (CAS) agar medium [35] was added and further incubated. The siderophore production capacity (Sid) was determined by the formation of an orange-colored halo around the bacterial culture. For positive siderophore production activity, the halo area (mm^2) was measured. The phosphate and zinc solubilizing capacities were tested using NBRIP [36] and TRIS-minimal [37] agar medium, respectively. For this purpose, the pellet was obtained from bacterial liquid culture, previously grown in liquid TY medium on a rotary shaker at 28 °C for 24 h up to the exponential growth

phase (OD_{600} = 0.5). The samples were centrifuged at 6000 rpm for 5 min, washed twice, and resuspended in 100 μL $MgCl_2$ 10 mM solution. Aliquots of bacterial suspensions containing 1×10^6 colony forming units (CFU/mL) were inoculated by triplicate on NBRIP and TRIS-minimal agar medium and incubated at 28 °C for 5 days. The phosphate solubilizing index (SI–P) was calculated as SI–P = A/B where A is the diameter of the colony plus the cleared area around it and B is the colony diameter [38], while the Zinc solubilization (ZnSI) was calculated ZnS(%) = (A/B) X 100, where A is the diameter of solubilization halo plus colony and B is the diameter of the colony [37].

2.7. Rhizospheric soil characterization

The remaining rhizospheric soil samples were analyzed through the standardized protocols of the Soil and Water Research Laboratory services of INDESCES (UNTRM, Peru). The samples, previously dried and pulverized, were used for Electric conductivity (EC) and pH determination in a soil/water suspension (1:5; w/v) using a Thermo Scientific Orion Star A215 pH/conductivity meter. The soil organic nitrogen (SON) was determined using the Walkley and Black test [39]. Phosphorus was obtained by the modified Olsen method and measured by atomic absorption photometry [40]. Cadmium (Cd^{2+}) concentrations were determined by atomic emission photometry (Agilent Technologies 4100 MP-AES) and Potassium (K^+) by ammonium acetate 1 N saturation method.

2.8. Diversity of bacterial community

The diversity analysis was performed using the *microeco* package of the R environment [41]. Alpha diversity indices: Shannon, Simpson, ACE, and Faith's phylogenetic diversity (PD), were calculated to assess the richness and distribution of species in the samples. The comparison of rhizosphere bacterial communities between sample sites (beta-diversity) was performed by computation of Bray-Curtis and Jaccard distances. Permutational multivariate analysis of variance (PERMANOVA) was applied to detect differences in rhizosphere bacterial communities between sites. Patterns in beta diversity were represented using Principal Coordinates Analysis (PCoA). To identify the effect of the soil physico-chemical properties on the distribution of the bacterial community, a distance-based redundancy analysis (RDA) was performed on the Bray-Curtis distance matrix. The integrative phylogenetic analysis was carried out with the *picant*e package also for R environment [42]. The descriptive Blomberg's K statistic [43] was used to measure the phylogenetic signal in trait association.

2.9. Data analysis

For the removal efficiency, siderophore production, and zinc solubilization assays, one-way ANOVA (analysis of variance) tests were conducted, followed by Tukey's multiple-comparison test as a post hoc analysis. When departure from the homogeneity of residues and/or normality was detected, a data transformation procedure (Normal Score) was applied. The phosphate solubilization test was performed using the Kruskal-Wallis non-parametric test. In all cases, different letters indicate statistically significant differences (p-value $\leq$0.05). Both ANOVA and Kruskall-Wallis tests were performed using the Infostat statistical software [44].

3. Results

3.1. Cacao rhizosphere soils characterization and bacteria isolation

After performing serial dilutions, 180 bacterial strains were isolated from cacao tree rhizospheres grown in three districts of Bagua province (Table 1, Supplementary Table S1). These strains were able to grow after 24 h of incubation at 28 °C. From this group, 90 representative strains (equal proportion per sampling site) were selected for further analysis. Following bacterial isolation, the

Table 1
Soil physicochemical characteristics of the sampling sites.

Sampling site	Location	pH	EC (dS/m)	SON (%)	P (ppm)	K (ppm)	Cd (ppm)	CFU/g
Cajaruro-a	5° 43′09.18″ S – 78° 20′58.61″ W	7.31 ± 0.14	0.017 ± 0.001	0.38 ± 0.03	3.98 ± 0.93	327.61 ± 40.18	<0.005	$2.8 \pm 1.3\ 10^5$
Cajaruro-b	5° 43′43.98″ S – 78° 20′59.06″ W	7.23 ± 0.03	0.018 ± 0.002	0.36 ± 0.07	6.26 ± 2.38	297.72 ± 23.44	<0.005	$2.03 \pm 1.2\ 10^5$
La Peca-a	5° 37′44.80″ S – 78° 25′07.58″ W	5.96 ± 0.38	0.013 ± 0.006	0.39 ± 0.06	2.93 ± 0.57	781.28 ± 194.35	<0.005	$0.7 \pm 0.6 \times 10^5$
La Peca-b	5° 37′44.80″ S – 78° 25′07.57″ W	6.33 ± 0.24	0.014 ± 0.004	0.36 ± 0.06	4.91 ± 4.0	643.49 ± 365.56	<0.005	$1.2 \pm 1.2 \times 10^5$
Imaza-a	5° 10′31.23″ S – 78° 17′10.57″ W	5.66 ± 0.53	0.012 ± 0.004	0.37 ± 0.00	3.25 ± 1.22	157.99 ± 108.3	1.35 ± 0.15	$2.5 \pm 1.2 \times 10^5$
Imaza-b	5° 10′17.83″ S – 78° 17′14.44″ W	5.7 ± 0.16	0.012 ± 0.001	0.29 ± 0.09	5.56 ± 3.4	151.93 ± 58.44	<0.005	$1.3 \pm 0.5 \times 10^5$

The physicochemical determination was performed as is described in section 2.5. pH: Potential of hydrogen; EC: Electric conductivity; SON: Soil organic nitrogen; P: Phosphorus: K: Potassium; CFU/g: Colony forming unit per gram.

remaining rhizosphere soil samples were characterized according to their physicochemical properties. The samples exhibited pH values ranging from moderately acidic (6.1–6.5) to neutral (6.6–7.3). The most acidic were those from the Imaza district (Table 1). Additionally, all samples showed to be non-saline soils (<1 dS/m) with low levels of Phosphorus (P) available (<10 ppm). The

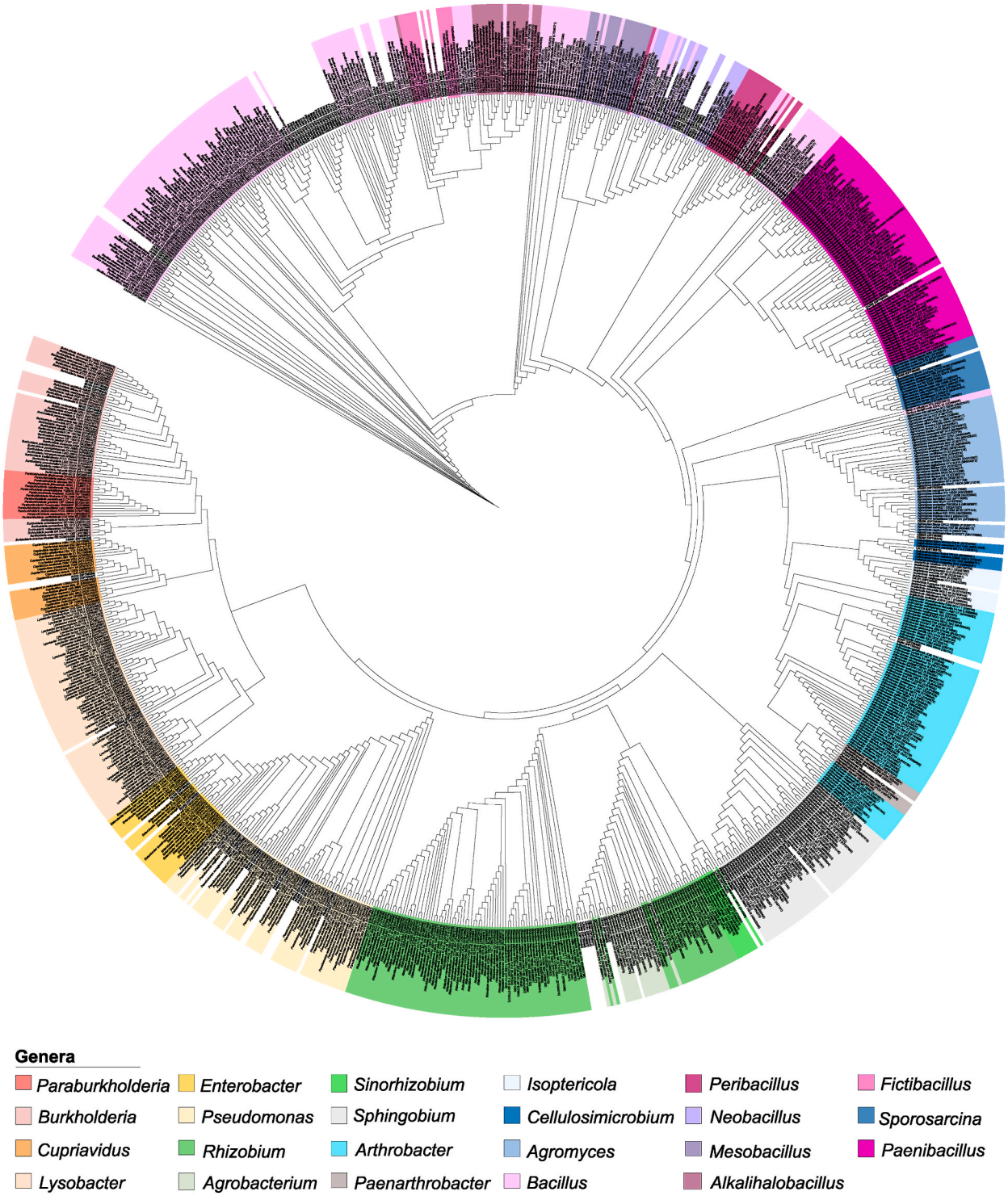


Fig. 1. Maximum likelihood phylogenetic tree based on 16S rRNA sequences showing the classification of rhizospheric bacterial isolates. The reference sequences (colored) can be distinguished by genus as indicated in the legend. The isolates are shown without a colored background. The accession numbers are indicated in parentheses as well as in Table 2 and Supplementary Table S2.

potassium (K) levels ranged from medium (150–250 ppm) to high levels (250–800 ppm), and the SOM levels ranged between 0.29 and 0.39%. Furthermore, considering the importance of cadmium (Cd) for cacao soils, Cd concentration was measured. Non-detectable levels were found in the samples (<0.005 ppm), except for the Imaza-b sampling site (1.35 ± 0.15 ppm) (Table 1).

3.2. Bacterial phylogenetic identity

The phylogenetic identification was conducted by comparing the 16S rRNA amplicons of 88 bacterial strains with good-quality sequences (>1000 pb) to representative reference sequences of type strains of 23 genera belonging to *Bacillaceae* (*Fictibacillus*, *Neobacillus*, *Alkalihalobacillus*, *Paenibacillus*, *Peribacillus*, *Mesobacillus*, *Bacillus*), *Promicromonosporaceae* (*Isoptericola*, *Cellulosimicrobium*), *Pseudomonadaceae* (*Pseudomonas*), *Rhizobiaceae* (*Rhizobium*, Sinorhizobium, *Agrobacterium*), *Micrococcaceae* (*Arthrobacter*, *Paenarthrobacter*), *Enterobacteriaceae* (*Enterobacter*), *Burkholderiaceae* (*Cupriavidus*, *Burkholderia*, *Paraburkholderia*), *Lysobacteraceae* (*Lysobacter*), *Microbacteriaceae* (*Agromyces*), *Sphingomonadaceae* (*Sphingobium*) and *Caryophanaceae* (*Sporosarcina*) bacterial families (Fig. 1, Supplementary table 2). These genera were selected through preliminary comparison with RDP and NCBI databases. To better understand the diversity between and within families, the reference sequences were colored by genus. The query bacterial sequences are shown without a colored background.

A total of 944 sequences were analyzed and stronger bootstrap values were obtained. As is shown in Figs. 1, 23 distinct clades were established. Furthermore, non-polyphyletic clades were observed, except for *Peribacillus*, *Neobacillus,* and *Sinorhizobium* genera which contained some *Bacillus* (*Bacillus massiliogorillae* G2, *B. massilioanorexius* AP8, *B. testis* SIT10 and *B. ferrooxidans* YT3) or *Rhizobium* strains (*Rizobium pongamiae* VKLR 01). According to the phylogenetic tree, 29 isolates belong to the *Bacillus* genus. Strains PB105, PA204, PA203, IB305, and PB103 clustered with *Bacillus mobilis* MCCC 1A05942, *B. marcorestinctum* LQQ, *B. toyonensis* BCT 7112, *B. paranthracis* MCCC 1A00395, *B. proteolyticus* MCCC 1A00365, *B. fungorum* 17 SMS 01 and *B. wiedmannii* FSL W8-0169, while the strains IA204, IA102, and IA308 did it with *B. mojavensis* NRRL B 14698, *B. halotolerans* DSM 8802, *B. nakamurai* NRRL B 41091 and *B. inaquosorum* NRRL B 23052; both with 96% of bootstrap. The strains PA103, PA104, IB304, PB107, and PB302 were found closely related to *B. zanthoxyli* 1433 at 100% of bootstrap. Similar results were observed for IA202, IA203, IA205, IA210, PB104, IB201, IB203, IB204, IB206, IB301, PA101, PA201, PB203, PA302, PA303, PA305, and CA210 which clustered with various *Bacillus* species; all with bootstrap values over 68%.

Other members of the *Bacillaceae* family were also found. For example, the strains CA202, IA101, CA104, and CA201 grouped with *Fictibacillus arsenicus* Con-a-3, *F. barbaricus* V2 BIII_A2, *F. phosphorivorans* CCM 8426, *F. halophilus* AS8, *F. nanhaiensis* JSM 082006, *F. aquaticus* GDSW-R2A3, *F. iocasae* S38 at 96% of bootstrap while the strains IB303, PB201 and PB101 were found with *Peribacillus muralis* LMG 20238, *P. simplex* DSM 1321 and *P. butanolivorans* K9, at 99% of bootstrap.

The strains CB301, CB106, CB302, and CB205 were found in a single clade with *Neobacillus niacini* NBRC 15566 while the strains PA301, PA304, and IB202 with *N. drentensis* NBRC 102427, *N. bataviensis* NBRC 102449, *N. soli* LMG 21838, *N. novalis* NBRC 102450 and *N. vireti* NBRC 102452, with bootstrap values of 58% and 44%, respectively. The strain CA103 was also found within the *Neobacillus* clade. Only the strain CA303 was found inside the *Paenibacillus* clade, closely related to *P. hordei* RH N24 and *P. kyungheensis* DCY 88 with a bootstrap value of 98%.

Twelve strains were classified in the *Pseudomonas* clade. The strains PA105, CA102, and CB305 clustered with *Pseudomonas oryzae* WM3, *P. sagittaria* CC OPY 1, and *P. aeruginosa* DSM 50071, while the strains PA205, PB304 y PB301 were found with *P. koreensis* Ps-9 14, *P. moraviensis* CCM 7280 and *P. kribbensis* 46 2, with bootstrap values of 95% and 98%, respectively. The strains PB204 and PB303 grouped with *P. wadenswilerensis* CCOS 864 and *P. donghuensis* HYS, while IB104 and IA306 with *P. soli* F 279 at 99 and 94%, respectively. Similarly, the strain PB202 was found closely related to *P. chlororaphis* DSM 50083 with a bootstrap value of 97%. Moreover, the strain PB305 was grouped with several *Pseudomonas* strains, with a bootstrap value of 92%.

A total of 9 bacterial strains clustered in the *Rhizobiaceae* family clade. Specifically, the strains CA304, CA301, CA302, CB101, CB102, CB105, and CB103 grouped with *Agrobacterium salinitolerans* YIC 5082, *A. pusense* NRCPB10, *Rhizobium pongamiae* VKLR_01 and *A. fabrum* C58 with a bootstrap value over 86%, while the strain PB206 and CA203 grouped in a separate clade with several *Sinorhizobium* strains at 89% of bootstrap.

For the *Burkholderiaceae* family, 7 strains showed to belong to *Cupriavidus* and *Burkholderia* genera. The strains CB201, IA302, and IB302 clustered with *Cupriavidus oxalaticus* DSM 1105, *C. yeoncheonensis* DCY 86, *C. basilensis* DSM 11853, *C. numazuensis* TE26, *C. laharis* 1263a and *C. pinatubonensis* 1245 with bootstrap values over 96%, while the strains IB102, IB105, IB106, and IB103 with *Burkholderia catarinensis* 89, *B. ambifaria* AMMD, *B. diffusa* R15930, *B. contaminans* I2956, *B. paludis* Msh1 and *B. arboris* R 24201 with bootstraps values over 91%.

In relation to *Micrococcaceae, Microbacteriaceae and Enterobacteriaceae* families, the strains PA102, PA202 were found closely related to *Enterobacter huaxiensis* 90008, *E. cancerogenus* LMG 2693 and *Enterobacter soli* LF7 with bootstrap values over 91%. The strains IA304 and IA307 were found in a single clade with several *Arthrobacter strains* at 78% and the strains CB202, CB203 CB204, and CB303 were found with *Agromyces tropicus* CM9-9, *A. archimandritae* G127ATT, *A. marinus* H23 8, *A. luteolus* IFO 16235, *A. subtropicus* IY107 56, *A. flavus* CPCC 202695, *A. aurantiacus* YIM 21741, *A. mediolanus* DSM 20152, *A. soli* MJ21, *A. ulmi* XIL01, *A. protactiae* FW100 M 8, and *A. indicus* NIO 1018 at 80% of bootstrap.

In addition, singular isolates per genus were also found. For example, only the strain IA201 was located in a single clade with *Sphingobium scionense* WP01 and *S. yanoikuyae* GIFU 9882 at 100% of bootstrap, CB304 was found with *Lysobacter cavernae* YIM C01544 and *L. tabacisoli* C8 1 at 99% of bootstrap, CA205 grouped with *Paenarthrobacter ilicis* DSMZ 20138 at 98% of bootstrap, CA101 grouped with *Isoptericola nanjingensis* H17 at 100% of bootstrap, CA105 was found grouped with several *Cellulosimicrobium* strains at 84% of bootstrap and CA305 was found closely related to *Sporosarcina thermotolerans* CCUG 53480 at 85% of bootstrap.

3.3. Rhizospheric Bacterial diversity and composition

As it was shown in the previous section, based on the phylogeny of 16S sRNA amplicons, 88 bacterial strains belonging to 19 different genera of the 10 bacterial families (*Promicromonosporaceae*, *Pseudomonadaceae*, *Bacillaceae*, *Rhizobiaceae*, *Micrococcaceae*,

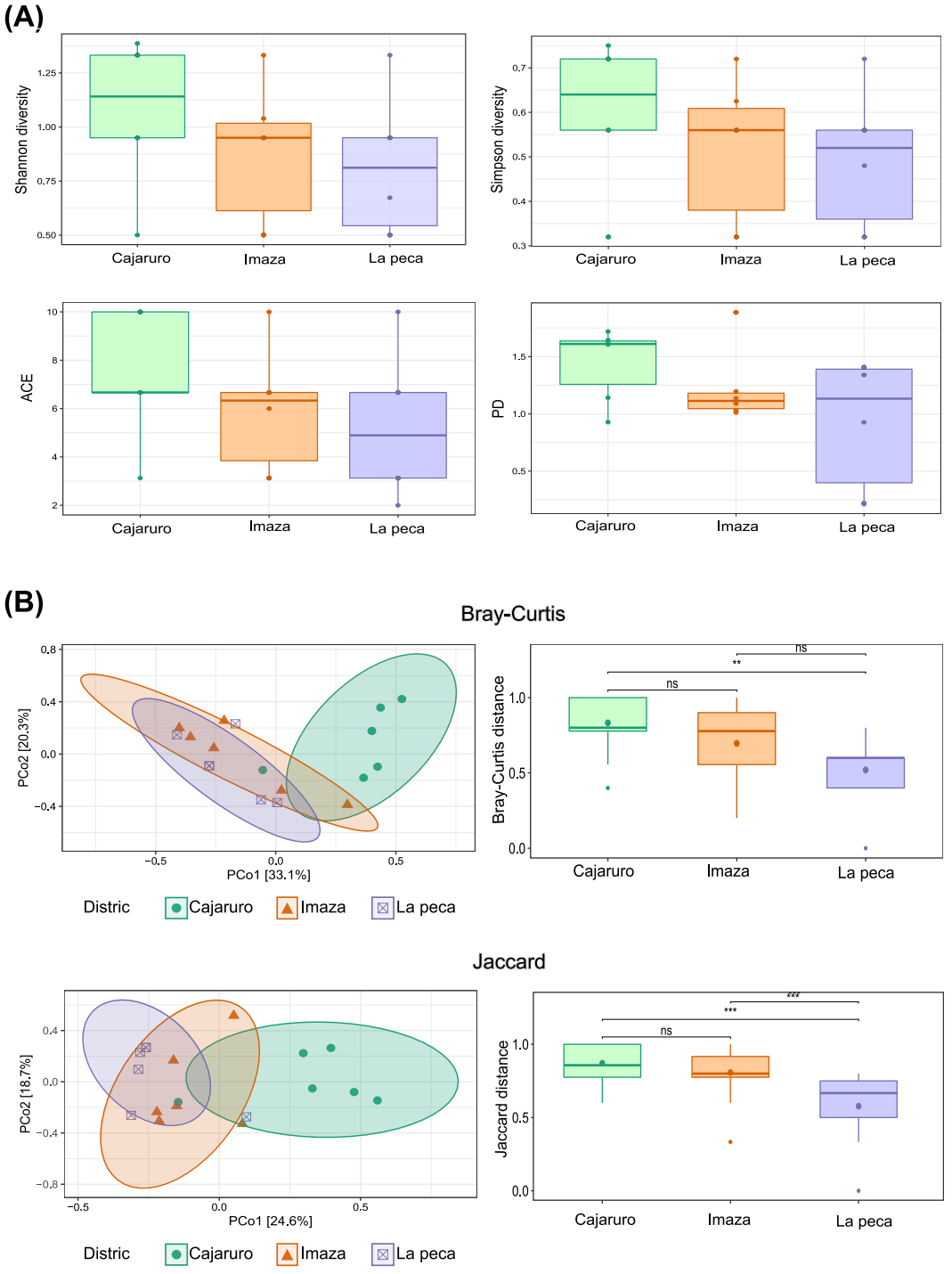


Fig. 2. (A) Boxplot of Shanon, Simpson ACE, and PD indexes of diversity calculated for 18 samples coming from Cajaruro, La Peca, and Imaza districts. (B) Principal coordinate analysis (PCoA) plots to display similarity relations among the bacterial rhizosphere communities, based on Bray-Curtis and Jaccard distances. For further analysis, each PCoA plot is accompanied by a boxplot graph of the Wilcoxon test (On the right). The significance is shown as follows: p-value ≤0.001(**), p-value ≤0.0001 (***), and non-significant (ns).

Enterobacteriaceae, *Lysobacteraceae*, *Microbacteriaceae*, *Sphingomonadaceae,* and *Caryophanaceae*) were found. Although this could suggest a high diversity status, proper diversity metrics were required. For this purpose, the strain's genus identities were used as a taxonomic unit. For intrinsic analysis, the alpha diversity was analyzed. These measurements revealed similar characteristics, among the districts. Non-parametric Kruskall-Wallis test showed non-significant differences for Shanon, Simpson, ACE, and PD indices (Fig. 2A), suggesting similar species richness and equitability between them. On the contrary, the beta diversity analysis showed divergence in the bacterial communities among the districts. According to Bray-Curtis and Jaccard distances (Fig. 2B), the samples are grouped by sampling area. PCoA plots revealed a visual separation of the samples from the Cajaruro district compared with La Peca and Imaza. This clustering was statistically supported by the PERMANOVA test (999 permutations, p-value $\leq$0.002 and p-value $\leq$0.003, respectively). For further analysis of the distances among the districts, the Wilcoxon test was conducted. It revealed the significant separation of the Cajaruro district (Fig. 2B).

A redundancy analysis (RDA) was performed, to explore the association between the bacterial distribution and soil Physicochemical variables. According to the RDA, the 55.8% variability was explained by RDA1 (p-value = 0.013, permutation test), where only the pH level in the soil was found statistically significant (p-value = 0.001, permutation test) (Fig. 3).

The communities displayed variability in the taxonomic composition according to the sample site. As is shown in Fig. 4A, the taxonomy distribution differed among the districts. Although there was a strong dominance of the *Bacillaceae*, their distribution and dominance varied according to the sampling area. In particular, La Peca and Imaza districts exhibited a stunning dominance of *Bacillus* strains while *Agrobacterium* and *Neobacillus* mainly dominated Cajaruro. Furthermore, it showed the presence of singular isolates of several less abundant bacterial taxa. This trend is also appreciated in Fig. 4B, where the unique and shared taxa are shown. The exclusive genera per district were *Enterobacter* in La Peca; *Sphingobium*, *Arthrobacter*, and *Burkholderia* in Imaza; and *Isoptericola*, *Cellulosimicrobium*, *Paenarthrobacter*, *Agrobacterium*, *Agromyces*, *Neobacillus*, and *Lysobacter* in Cajaruro. Meanwhile, the shared taxa among the districts were *Fictibacillus* and *Cupriavidus* for Cajaruro and Imaza; *Rhizobium* for Cajaruro and La Peca; and *Peribacillus* for La Peca and Imaza. Only *Bacillus*, *Pseudomonas,* and *Neobacillus* strains were found in the three districts; these latter composed 55.7% of the total population.

3.4. Biocontrol of *M. roreri* capacity and cadmium removal efficiency

For functional characterization, all the identified strains (88) were tested to evaluate their potential to combat major cacao production issues, under *in vitro* conditions (Table 2). They were evaluated according to their antagonism index against *M. roreri* and Cd removal efficiency. These traits were primarily selected due to their relevance to the major limiting crop factor affecting cacao production. Interestingly, the antagonisms against *Moniliophthora roreri*, was the most dominant trait among the isolates. The antagonism index (AI) is shown in Fig. 5A. The results were classified as follows: Less than 25% (<25), between 25 and 50% (25–50), between 51 and 75 % (50–75), and more than 75% of AI (>75). On 7 days of the confrontation, 56.8% of the bacterial population showed antagonisms index (AI-7) values greater than 75%. These bacteria were members of *Bacillus, Pseudomonas*, *Burkholderia*, *Neobacillus*, *Peribacillus*, *Agrobacterium*, *Agromyces*, *Sinorhizobium*, *Fictibacillus*, *Paenarthrobacter*, *Cupriavidus*, *Lysobacter*, *Enterobacter*, *Sphingobium* and *Sporosarcina* genera with a strong dominance of *Bacillus* followed by *Enterobacter* and *Agrobacterium* strains. Furthermore, 15 days after the confrontation, the bacteria with antagonism activity greater than 75% increased up to 68.2% of the population (Fig. 5A). On

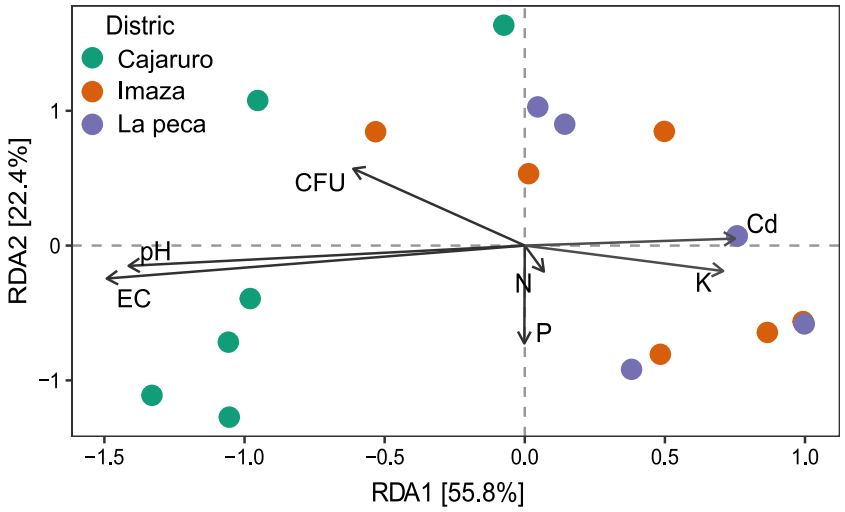


Fig. 3. Scatterplot of the two first axis resulting from Redundancy Analysis (RDA) for culturable bacteria associated with native cacao in Cajaruro (green), La Peca (purple), and Imaza (orange) districts and selected physicochemical properties of the Soil. The samples are represented with filled circles and the physicochemical properties are indicated by arrows, as follows: EC (electric conductivity), pH (potential of hydrogen); SON (soil organic nitrogen), P (phosphorus), Cd (cadmium), K (potassium) and CFU (colony-forming unit). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

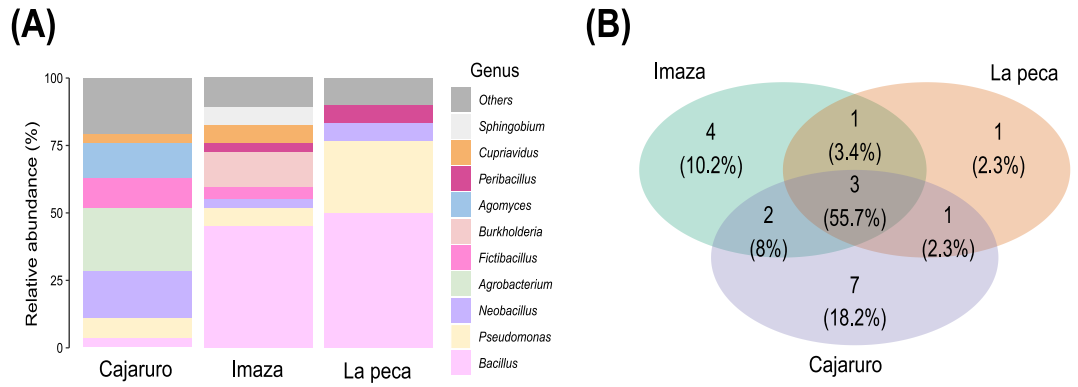


Fig. 4. (A) Relative abundance of rhizospheric culturable bacteria associated with *Theobroma cacao* by districts. Only the ten most abundant genera are displayed, distinguishable by colors as is indicated in the legend. The less abundant genera are grouped under "Others". (B) Venn Euler diagram illustrating taxa unique and shared among Districts. The percentage of the community population that they represent is indicated in the graphic. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the contrary, only the strains *Burkholderia* IB103, IB102, IB105, and IB106 (Fig. 5E) were capable remove cadmium from the medium or cadmium removal capacity at 100 and 200 ppm of $CdCl_2$ in the medium, non-significant differences were found. The detailed measurements and statistical comparisons of the traits are shown in Table 2.

3.5. Plant growth promoting rhizobacteria attributes

Out of 88 strains, 18 (20.45%) were able to solubilize the inorganic phosphate of the NBRIP agar medium. These bacteria were members of the *Pseudomonas* (6), *Bacillus* (5), *Burkholderia* (4), *Enterobacter* (2), and *Sinorhizobium* (1) genera; and showed SI–P values over 1.29. To them, the strains *Bacillus* PA101 and *Pseudomonas* PB305 exhibited the higher significant phosphate solubilization performance (1.53 ± 0.03 and 1.54 ± 0.03, respectively) (Fig. 5B).

The siderophore production test revealed only 12 strains (13.63%) produced siderophore under *in vitro* conditions. Most of them belong to *Pseudomonas* (5), followed by *Burkholderia* (4), *Bacillus* (2), and *Cupriavidus* genera. As shown in Fig. 5C, the strain *Pseudomonas* PB204 exhibited the highest siderophore production followed by *Pseudomonas* PB303. The Zinc solubilization capacity (ZnS) was also assessed. Interestingly, only the strains Burkholderia IB103, IB102, IB105, and IB106 genus (Fig. 5D) were capable of solubilizing zinc from the medium. In particular, the strain Burkholderia IB102 showed significant zinc solubilization capacity (147 ± 2.09) compared with IB103, IB105, and IB106.

Complementary, integrative phylogenetic-functional analysis was assessed. For this purpose, the descriptive Blomberg's K statistic was calculated on the traits. Descriptive K statistics indicated low values of association between phylogeny and traits: phosphate solubilization: 0.33; siderophores: 0.32 and zinc solubilization: 0.31 (Supplementary Figure S1). In accordance with the beta diversity and taxonomic composition, the bacterial community was distributed according to the PGPR properties of the isolates among the districts. La Peca and Imaza exerted several bacteria holding at least two PGP capacities (*Bacillus*, *Enterobacter*, *Pseudomonas*, *Rhizobium,* and *Burkholderia*), while Cajaruro only displayed a single strain with antagonistic and siderophore-producer capacities (*Agrobacterium* CB101). This evidenced an unequal functional distribution of traits suggesting not only the variable potential of the rhizospheric soils of these districts as a source of PGPR but also their soil functional status. Moreover, this tendency matches with the microbial community variations among the districts found in section 3.3.

4. Discussion

In this work, we explored the diversity and the functional potential of rhizosphere-culturable bacteria associated with amazonian fine-flavor cacao. Out of 180 rhizospheric bacterial isolates, 88 strains were used for phylogenetic identification to unravel the bacterial community composition. The results evidenced a strong predominance of *Bacillaceae* members. Several isolates of *Bacillus*, *Paenibacillus*, *Fictibacillus*, *Peribacillus*, and *Neobacillus* genera were also found. This agrees with previous reports, which have shown the association of *Bacillus* with *Theobroma cacao* [14,23,45–47]. Moreover, the involvement of *Bacillus* in the rhizosphere has been demonstrated through plant growth-promotion attributes such as indole-3-acetic acid (IAA) production, phosphate solubilization, and biofilm development [48]. P revious reports were not found about the presence of *Fictibacillus*, *Peribacillus*, and *Neobacillus* associated with the cacao rhizosphere. However, this could be associated with the recent reorganization of the *Bacillus* genus and the novel proposal as separate genera [49].

In support of our findings, *Pseudomonas*, *Burkholderia*, *Cupriavidus*, and *Enterobacter* bacteria can also be found living in association with cacao roots [14,26,50–53]. P revious reports were not found about the isolates of *Agrobacterium*, *Sinorhizobium*, *Sphingobium*, *Isoptericola*, *Celulosimicrobium*, *Sporosarcina*, *Paenarthrobacter*, *Arthrobacter*, and *Lysobacter* genera. However, these bacteria have been found inhabiting the rhizosphere of other plant hosts [54–60].

Table 2
Native culturable bacterial isolates and functional characterization.

Strain	Site	Identity	AN	AI-7 (%)	AI-15 (%)	ZnS (%)	SI–P	Sid (mm^2)	RE -100 ppm (%)	RE - 200 ppm (%)
CA101	Cajaruro	*Isoptericola*	OP739368	73.75	91.32	–	–	–	–	–
CA102	Cajaruro	*Pseudomonas*	OP739369	77.80	64.75	–	–	–	–	–
CA103	Cajaruro	*Bacillus*	OP739370	18.54	2.49	–	–	–	–	–
CA104	Cajaruro	*Fictibacillus*	OP739371	12.02	4.07	–	–	–	–	–
CA105	Cajaruro	*Cellulosimicrobium*	OP739372	26.49	33.57	–	–	–	–	–
CA201	Cajaruro	*Fictibacillus*	OP739354	79.87	92.18	–	–	–	–	–
CA202	Cajaruro	*Fictibacillus*	OP739350	77.90	91.68	–	–	–	–	–
CA203	Cajaruro	*Rhizobium*	OP739351	78.01	88.76	–	–	–	–	–
CA205	Cajaruro	*Paenarthrobacter*	OP739352	78.43	91.14	–	–	–	–	–
CA210	Cajaruro	*Bacillus*	OP739336	77.85	91.25	–	–	–	–	–
CA301	Cajaruro	*Agrobacterium*	OP739394	82.89	88.95	–	–	–	–	–
CA302	Cajaruro	*Agrobacterium*	OP739395	82.60	79.28	–	–	–	–	–
CA303	Cajaruro	*Bacillus*	OP739383	73.79	90.96	–	–	–	–	–
CA304	Cajaruro	*Agrobacterium*	OP739337	81.80	86.18	–	–	–	–	–
CA305	Cajaruro	*Bacillus*	OP739396	79.66	86.57	–	–	–	–	–
CB101	Cajaruro	*Agrobacterium*	OP739410	79.61	82.92	–	–	–	–	–
CB102	Cajaruro	*Agrobacterium*	OP739411	75.73	78.39	–	–	–	–	–
CB103	Cajaruro	*Agrobacterium*	OP739413	82.24	86.19	–	–	–	–	–
CB105	Cajaruro	*Agrobacterium*	OP739353	27.15	45.10	–	–	–	–	–
CB106	Cajaruro	*Neobacillus*	OP739355	83.83	80.21	–	–	–	–	–
CB201	Cajaruro	*Cupriavidus*	OP739338	85.06	85.50	–	–	1.37 ± 0.21^{a}	–	–
CB202	Cajaruro	*Agromyces*	OP739384	75.70	80.38	–	–	–	–	–
CB203	Cajaruro	*Agromyces*	OP739385	27.72	31.86	–	–	–	–	–
CB204	Cajaruro	*Agromyces*	OP739386	36.12	35.92	–	–	–	–	–
CB205	Cajaruro	*Neobacillus*	OP739397	11.46	4.13	–	–	–	–	–
CB301	Cajaruro	*Neobacillus*	OP739339	73.14	87.66	–	–	–	–	–
CB302	Cajaruro	*Neobacillus*	OP739387	79.26	80.01	–	–	–	–	–
CB303	Cajaruro	*Agromyces*	OP739388	81.40	92.00	–	–	–	–	–
CB304	Cajaruro	*Lysobacter*	OP739340	80.34	91.64	–	–	–	–	–
CB305	Cajaruro	*Pseudomonas*	OP739389	80.05	90.70	–	–	–	–	–
PA101	La peca	*Bacillus*	OP739356	80.98	92.18	–	1.53 ± 0.03^{d}	2.77 ± 0.21^{bcd}	–	–
PA102	La peca	*Enterobacter*	OP739345	80.19	92.51	–	1.5 ± 0.03^{cd}	–	–	–
PA103	La peca	*Bacillus*	OP739346	80.99	91.70	–	–	–	–	–
PA104	La peca	*Bacillus*	OP739347	79.91	82.45	–	1.40 ± 0.03^{abcd}	–	–	–
PA105	La peca	*Pseudomonas*	OP753558	80.56	92.47	–	1.48 ± 0.03^{bcd}	2.62 ± 0.21^{bcd}	–	–
PA201	La peca	*Bacillas*	OP739357	69.24	91.77	–	–	–	–	–
PA202	La peca	*Enterobacter*	OP739358	68.69	91.31	–	1.41 ± 0.03^{abcd}	–	–	–
PA203	La peca	*Bacillus*	OP739348	73.27	91.57	–	1.29 ± 0.04^{a}	–	–	–
PA204	La peca	*Bacillus*	OP739402	70.11	91.91	–	1.36 ± 0.03^{abcd}	–	–	–
PA205	La peca	*Pseudomona*	OP753559	48.68	35.34	–	–	–	–	–
PA301	La peca	*Neobacillus*	OP739359	79.12	90.48	–	–	–	–	–
PA302	La peca	*Bacillus*	OP739360	80.73	91.40	–	–	–	–	–
PA303	La peca	*Bacillus*	OP739407	75.45	90.75	–	–	–	–	–
PA304	La peca	*Neobacillus*	OP739361	81.92	88.77	–	–	–	–	–
PA305	La peca	*Bacillus*	OP739362	43.29	29.95	–	–	–	–	–
PB101	La peca	*Peribacillus*	OP739400	69.19	61.09	–	–	–	–	–
PB103	La peca	*Bacillus*	OP739401	77.42	58.02	–	–	–	–	–
PB104	La peca	*Bacillus*	OP739349	30.59	14.71	–	–	–	–	–
PB105	La peca	*Bacillus*	OP739341	81.12	58.66	–	–	–	–	–
PB107	La peca	*Bacillus*	OP739363	85.45	65.89	–	–	–	–	–
PB201	La peca	*Peribacillus*	OP739391	83.60	79.93	–	–	–	–	–
PB202	La peca	*Pseudomonas*	OP753563	70.34	85.71	–	–	–	–	–
PB203	La peca	*Bacillus*	OP739392	27.07	41.39	–	–	–	–	–
PB204	La peca	*Pseudomonas*	OP753564	79.01	90.84	–	1.38 ± 0.03^{abcd}	6.23 ± 0.21^{d}	–	–
PB206	La peca	*Rhizobium*	OP739408	80.14	91.25	–	1.30 ± 0.03^{ab}	–	–	–
PB301	La peca	*Pseudomonas*	OP753565	80.59	91.37	–	1.43 ± 0.03^{abcd}	–	–	–
PB302	La peca	*Bacillus*	OP739393	74.93	89.09	–	–	–	–	–

(*continued on next page*)

Table 2 (*continued*)

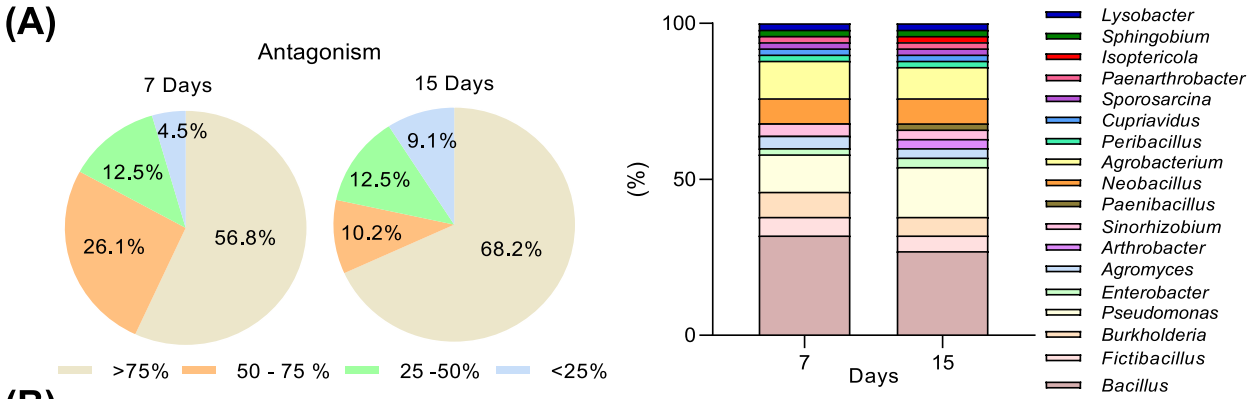
Strain	Site	Identity	AN	AI-7 (%)	AI-15 (%)	ZnS (%)	SI–P	Sid (mm^2)	RE -100 ppm (%)	RE - 200 ppm (%)
PB303	La peca	*Pseudomonas*	OP753567	74.93	89.09	–	1.37 ± 0.03^{abcd}	5.34 ± 0.21^{cd}	–	–
PB304	La peca	*Pseudomonas*	OP753566	74.93	89.09	–	–	–	–	–
PB305	La peca	*Pseudomonas*	OP753562	74.93	89.09	–	1.54 ± 0.03^{d}	2.07 ± 0.21^{abc}	–	–
IA101	Imaza	*Fictibacillus*	OP739378	79.31	91.74	–	–	–	–	–
IA102	Imaza	*Bacillus*	OP739379	79.79	88.85	–	1.37 ± 0.03^{abcd}	1.46 ± 0.21^{a}	–	–
IA110	Imaza	*Bacillus*	OP739399	69.93	45.67	–	–	–	–	–
IA201	Imaza	*Sphingobium*	OP739373	80.01	91.51	–	–	–	–	–
IA202	Imaza	*Bacillus*	OP739374	1.65	0.58	–	–	–	–	–
IA203	Imaza	*Bacillus*	OP739375	48.43	29.34	–	–	–	–	–
IA204	Imaza	*Bacillus*	OP739376	81.34	89.51	–	–	–	–	–
IA205	Imaza	*Bacillus*	OP739377	82.45	92.04	–	–	–	–	–
IA302	Imaza	*Cupriavidus*	OP739380	60.39	57.56	–	–	–	–	–
IA304	Imaza	*Arthrobacter*	OP739342	68.92	91.00	–	–	–	–	–
IA306	Imaza	*Pseudomonas*	OP753561	69.35	91.54	–	–	–	–	–
IA307	Imaza	*Arthrobacter*	OP739406	68.68	91.30	–	–	–	–	–
IA308	Imaza	*Bacillus*	OP739412	82.40	91.72	–	–	–	–	–
IB102	Imaza	*Burkholderia*	OP739364	85.86	92.15	147.83 ± 2.09^{b}	1.32 ± 0.03^{ab}	1.78 ± 0.26^{ab}	52.56 ± 2.96	47.45 ± 3.35
IB103	Imaza	*Burkholderia*	OP739365	86.18	92.30	130.77 ± 2.09^{a}	1.48 ± 0.03^{bcd}	1.73 ± 0.21^{ab}	53.15 ± 2.96	46.07 ± 3.35
IB104	Imaza	*Pseudomona*	OP753560	86.01	92.07	–	1.38 ± 0.03^{abcd}	1.97 ± 0.21^{ab}	–	–
IB105	Imaza	*Burkholderia*	OP739366	77.14	91.77	127.24 ± 2.09^{a}	1.32 ± 0.03^{ab}	1.80 ± 0.21^{ab}	51.07 ± 2.96	41.95 ± 3.35
IB106	Imaza	*Burkholderia*	OP739367	78.07	92.14	135.99 ± 2.09^{a}	1.33 ± 0.03^{abc}	2.73 ± 0.21^{bcd}	53.49 ± 2.96	41.77 ± 3.35
IB201	Imaza	*Bacillus*	OP739403	61.47	35.58	–	–	–	–	–
IB202	Imaza	*Neobacillus*	OP739390	47.78	58.96	–	–	–	–	–
IB203	Imaza	*Bacillus*	OP739398	50.09	10.76	–	–	–	–	–
IB204	Imaza	*Bacillus*	OP739405	48.22	23.06	–	–	–	–	–
IB206	Imaza	*Bacillus*	OP739404	53.93	19.10	–	–	–	–	–
IB301	Imaza	*Bacillus*	OP739343	77.90	91.94	–	–	–	–	–
IB302	Imaza	*Cupriavidus*	OP739381	51.89	30.20	–	–	–	–	–
IB303	Imaza	*Peribacillus*	OP739382	67.46	61.83	–	–	–	–	–
IB304	Imaza	*Bacillus*	OP739409	81.43	68.18	–	–	–	–	–
IB305	Imaza	*Bacillus*	OP739344	79.42	78.74	–	–	–	–	–

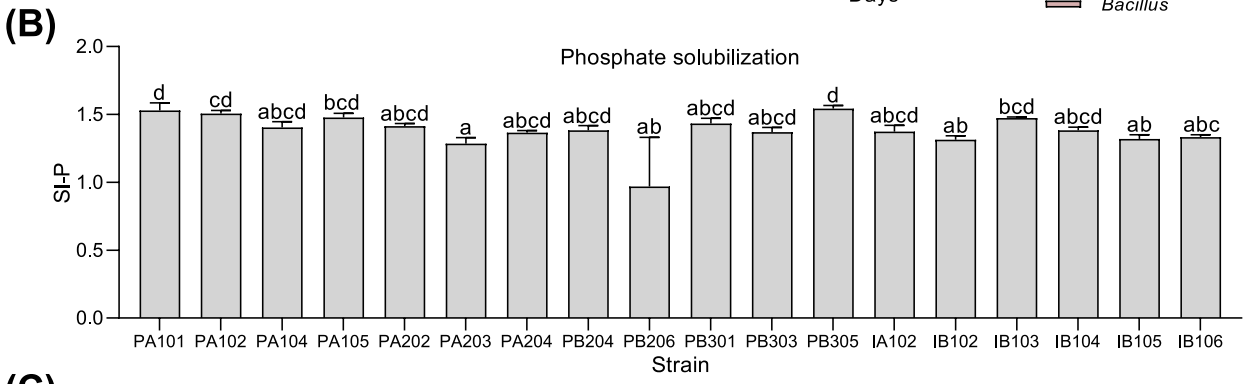
AN: Accession number; AI-7: Antagonism capacity index at 7 days after confrontation; A1-15: Antagonism capacity index at 15 days after confrontation; ZnS: Zinc solubilization; SI–P: Phosphate solubilization index; Sid: Siderophore production; RE-100 ppm: Cadmium removal efficiency at 100 ppm of CdCl2; RE-200 ppm: Cadmium removal efficiency at 200 ppm of CdCl2. For RE, Sid, and ZnS, one-way ANOVA (analysis of variance) tests were conducted, followed by Tukey's multiple comparison as post hoc analysis. The SI–P was analyzed using the Kruskal-Wallis non-parametric test. In all cases, different letters indicate statistically significant differences (p-value ≤ 0.05).

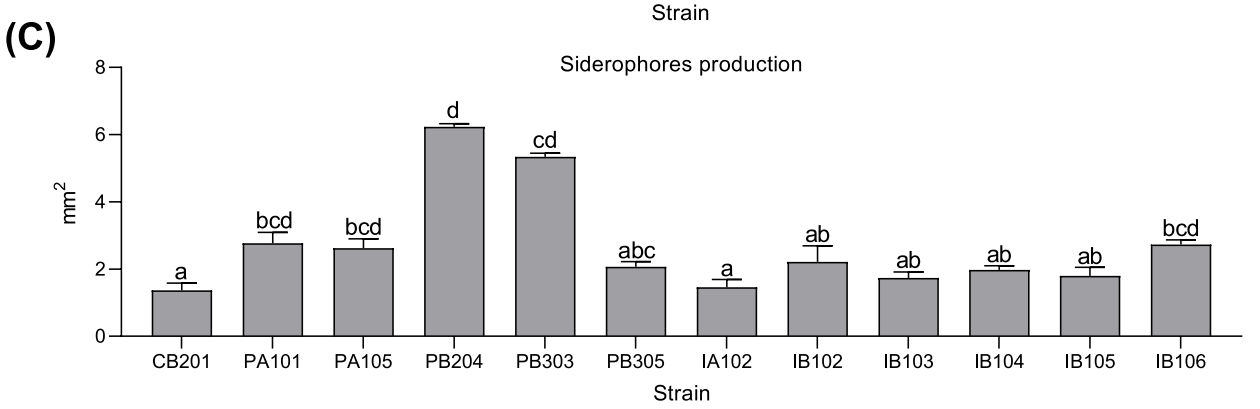
Although the conventional culturing method is a widespread technique, little is known about the diversity of culturable rhizospheric bacteria. For that reason, the bacterial population mentioned before was compared to perform a diversity analysis. According to alpha diversity, similar characteristics can be found among the districts. Non-significant differences were obtained for the Shanon, Simpson, ACE, and PD index, suggesting a similar richness and equitability of the bacterial taxes. Similar studies about cultured bacterial diversity in other important crops, such as *Thymus zygis* and *Zea mays* revealed a Shannon-wiener (H) index of 4.23 and 2.10, respectively, which are at least two-fold higher than ours (from 0.81 to 1.08) [61,62]. Although this suggests higher alpha diversity, those differences could be explained by the number of individuals analyzed, since phylogenetic diversity increases according to the sampling size [63].

In terms of relative abundance, *Bacillus* strains were the most abundant culturable bacteria in the cacao rhizosphere. This genus is highly culturable and numerous members are relevant for developing biobased approaches applicable to sustainable agriculture [64]. Moreover, it has been reported as the most abundant bacteria in other agroecosystems, like *Zea mays* rhizosphere [61], suggesting that members of the *Firmicutes* phylum are frequent in the rhizosphere of some important crops. The microbial community and its divergence has been well studied by numerous reports where it strongly supports the influence of the plant hosts as the main driver of the community composition and structure [20,65,66]. However, for culture-dependent isolates, the influence of the culture medium and conditions should also be considered as determinants of the characterization of culturable bacterial diversity.

According to distance matrix analysis, significant divergences were found in the bacterial community composition, particularly in the Cajaruro district compared with Imaza and La Peca. Furthermore, the major determinant of the bacterial distribution was the pH of the soil environment. In agreement, several studies have also assessed the effects of pH on microbial diversity and community structure [67,68]. Growing evidence supports the statement that soil pH is an important predictor of bacterial community composition and







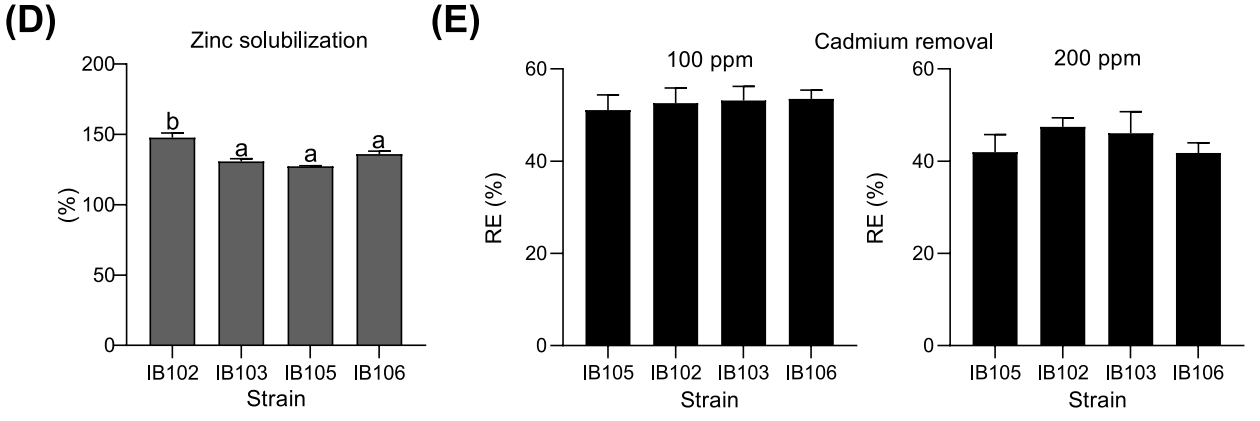


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Fig. 5. Functional Assessment of the Bacterial Isolates. (A) Pie-charts of the antagonistic index (AI) against *Moniliophthora roreri* at 7 and 15 days after direct confrontation. The results were categorized as follows: Less than 25% (<25), between 25 and 50% (25–50), between 51 and 75 % (50–75), and more than 75% of AI (>75). On the right, the stacked bar chart displays the bacterial genera of the isolates with AI greater than 75%. (B) Inorganic Phosphate Solubilization Index. (C) Siderophore Production. (D) Zinc Solubilization Efficiency, and (E) Cadmium Removal Efficiency. The bars represent the means of three replicates +/SE. For the removal efficiency, siderophore production, and zinc solubilization assays, one-way ANOVA (analysis of variance) tests were conducted, followed by Tukey's multiple comparison test as post hoc analysis. The phosphate solubilization test was performed using the Kruskal-Wallis non-parametric test. In all cases, different letters indicate statistically significant differences (p-value ≤0.05). The bar plots were done with GraphPad v.8.0.1. software.

diversity. The influence of pH level seems to be greater than that exerted by vegetation type or other physicochemical properties [69]. Moreover, in a recent meta-analysis involving 1235 global change factors (GCF), experimental observations have evidenced a significant effect on microbial alpha diversity, primarily driven by the changed soil pH. Interestingly, the authors also found a relationship between the soil pH level and alpha diversity [70]. The key role of pH in shaping microbial communities could be attributed to its intimate connection with microbial survival capacity. This includes the adaptive strategies required to maintain intracellular pH homeostasis, such as cation/proton antiporters, amino acid transport and metabolism, nucleotide transport and metabolism, cell wall/membrane/envelope biogenesis, and carbohydrate transport and metabolism [71–73]. The cultured depended method provides pure isolates to perform metabolic, genetic, and functional analyses and their further biotechnological application. In this regard, to assess the functional performance of the culturable bacterial community and identify promising bacteria, several *in vitro* phenotypic tests were evaluated. Special emphasis was placed on antagonism and cadmium removal testing, due to their close relationship with the major limiting crop production of cacao. The most dominant trait among the isolates was the antagonisms against *M. roreri*. This pathogen is the responsible agent of frosty pod rot in cacao, and potentially the most destructive disease affecting this crop [74,75]. Over half of the bacterial population showed antagonism index values greater than 75% at both 7 (56.8%) and 15 days (68.2%) of confrontations. These bacteria were members of *Bacillus, Pseudomonas*, *Burkholderia*, *Neobacillus*, *Peribacillus*, *Agrobacterium*, *Agromyces*, *Sinorhizobium*, *Fictibacillus*, *Paenarthrobacter*, *Cupriavidus*, *Lysobacter*, *Enterobacter*, *Sphyngobium*, *Sporosarcina* genera, with strong dominance of *Bacillus* followed by *Enterobacter* and *Agrobacterium* strains. While fungal agents have predominantly been studied for biological control against *M. roreri*, certain bacteria have also demonstrated antagonistic effects. Such is the case of several endophyte isolates. In line with our findings 26 *Bacillus* isolates showed antagonism capacity with a minimal pathogen growth inhibition of 28.6%. In addition, some of these isolates demonstrated antagonistic behavior against *M. perniciosa* and *Phytophthora capsici* [23]. Similar antagonistic effects were shown by Ref. [24]. The authors found several *Bacillus* bacterial isolates with the mycelial inhibition capacity of *M. roreri* and *M. perniciosa*. Moreover, these bacteria could produce biosurfactant compounds, which, at concentration exceeding 35 mg/L, reduced the total growth of vegetative mycelium of *M. roreri*. Similarly, the VOC produced by *B. pumilus* CFFSUR-B34, *B. muralis CFFSUR- B39,* and *Novosphingobium lindaniclasticum* CFFSUR-B36 inhibited *M. roreri* mycelium growth by over 35%, sporulation by over 81% and spore germination by over than 74% [76]. No previous antagonistic effects against *M. roreri* were found for the abovementioned remaining genera. However, the antimicrobial activity, of most of them, against other pathogens has been previously reported [77–84]. Only the *Burkholderia* isolates showed cadmium removal capacity. They could remove up to 53.49% of cadmium in the liquid medium. Some *Burkholderia* strains with variable Cd-removal capacities have also been isolated in association with the cacao rhizosphere. For example, the strain *Burkholderia* sp. NB10, evidenced a higher Cd-resistant capacity, being able to grow up to 140 mg/kg [53]. Similar results were seen for *Burkholderia* strain S-17, which also evidenced intracellular Cd accumulation capacity and 21% of Cd capture rate under *in vitro* conditions [26]. The capacity of *Burkholderia* to remove heavy metals has been widely studied [85–88]. Besides the intracellular bioaccumulation, other proposed mechanisms are the Cd-immobilization induced by phosphate precipitation and biosurfactant production [85,88].

Complementary, among the isolates, 18 strains could solubilize the inorganic phosphate of the NBRIP agar medium, whereas only 4 could solubilize zinc. These bacteria were identified as members of the *Pseudomonas*, *Bacillus*, *Burkholderia*, *Enterobacter*, and *Sinorhizobium* genera. Of these, only *Burkholderia* strains were able to zinc solubilization. Despite the scarce studies, some bacteria such as *Bacillus* and *P. aeruginosa* associated with cacao, have evidenced the capacity to solubilize phosphate under *in vitro* conditions [48,89]. No previous reports were found of phosphate or zinc solubilizing strains of *Burkholderia*, *Enterobacter*, and *Sinorhizobium* genera associated with cacao rhizosphere. Both phosphorus and zinc are essential macro and micronutrients, respectively, required for optimum plant growth. However, only a minor fraction of total P and Zn are present in soil solution as soluble. Those bacteria with the capacity to transform the inaccessible P or Zn share, as the major mechanism, the production of organic acids [90,91] Recent reviews have pointed out a wide range of bacteria including *Pseudomonas*, *Pantoea*, *Enterobacter*, *Serratia*, *Erwinia*, *Rahnella, Citrobacter*, *Burkholderia*, *Klebsiella,* and *Rhizobium* with this capacity [90–93]. These strains have been reported for increasing metal availability and their undeniable potential to replace conventional agricultural practices. Siderophore production capacity was also tested. Twelve strains exhibited the ability to produce siderophores under *in vitro* conditions. Most of these belong to *Pseudomonas*, followed by *Burkholderia*, *Bacillus*, and *Cupriavidus* genera. In concordance, a massive characterization of 185 bacilli, isolated from the rhizosphere soils of cocoa from various locations in southern states of India, evidenced that 20% of them were able to produce siderophore and hydrogen cyanide [50]. Similar results were found for three *Pseudomonas chlororaphis* strains associated with the cacao rhizosphere [51]. They could produce siderophores, and exert a high antagonism effect against *Phytophthora palmivora.* Similarly, strains of *Streptomyces albovinaceus, S. caviscabies, S. griseus, S. setonii, and S. virginiae,* initially selected by their capacity to inhibit *M. perniciosa*, could also to produce siderophore under *in vitro* conditions [94]. Interestingly, this dual behavior was also seen in this study. Siderophore-producing isolates were found to exert antagonistic effects against *M. roreri*. This could be explained by the relationship between the siderophore and the antagonistic effect.

It should be mentioned that isolates, *Burkholderia* strains IB102, IB103, IB105, and IB106, were the only strains that tested positive for every PGPR trait assessed. However, although these could be promising from a biotechnological perspective, it must be considered that the use of microorganisms is not a risk-free solution. In other words, in favor of biosafety, the potential biohazards associated with the usage of bacteria should be considered [95].

In addition, the isolates CB201, PA101, PA102, PA104, PA105, PA202, PA203, PA204, PB204, PB206, PB301, PB303, PB305, IA102, and IB104, also exhibited multiple PGPR behavior. According to the results, they tested positive for at least two PGP traits. This, along with the biosafety criteria already mentioned, made them potential candidates to be studied, either from an individual or community approach (e.g. synergistic bacterial consortium).

Integrative phylogenetic-functional analyses were also conducted. First, integrative phylogenetic signal analysis was performed to predict the relationship between the degree of phylogenetic relatedness and phenotypic response, to presume phylogenetic conservatism of the tested traits among isolates. This information can help identify markers for specific microbial taxa, apply phylogeny-based algorithms to predict unknown traits, and provide valuable ecological insights into the evolution of traits that impact ecosystems [96,97]. According to the descriptive K statistic, low values of association between phylogeny and siderophores production, phosphate, and zinc solubilization were found, suggesting a weak phylogenetic signal for these traits. That is to say, these properties for *Bacillus*, *Pseudomonas*, *Enterobacter,* and *Burkholderia,* have a small evolutionary component. Therefore, it is not expected that closely related taxonomic groups would express similar functional characteristics. Weak phylogenetic signals in bacteria could be explained by their genome plasticity and faster response mechanisms, which provide them with selected adaptive behavior imposed by the environments [98].

The phylogenetic signals found could be supported by the reports of several isolates displaying those phenotypic characteristics [90,91,99,100]. In particular, for *Pseudomonas*, the closest evidence about the conservatism of solubilization traits among the genera has been reported by Browne et al. (2009). However, since then, studies have mainly focused on the presence/absence of traits, little is still known about phylogenetic signals in closely related bacteria co-ocurring in the rhizosphere.

Finally, when the community was categorized based on the PGPR potential of the isolates, an unequal functional distribution was evident. Whereas La Peca and Imaza harbored several bacteria with at least two PGP capacities (*Bacillus*, *Enterobacter*, *Pseudomonas*, *Rhizobium,* and *Burkholderia*), Cajaruro only displayed a single strain with antagonistic and siderophore-producing capacities. This suggests not only the variable potential of the rhizospheric soils in these districts as a source of PGPR organisms but also their functional heterogeneity. This observation aligns with the significant community composition differences among the districts, suggesting that community structure likely influences functional roles exhibited in the rhizosphere.

In conlusion, the rhizosphere of the Amazonian native fine-flavor cacao from representative Peruvian productive districts (La Peca, Imaza, and Cajaruro) harbors a culturable bacterial community with similar richness and equitability (alpha diversity). However, despite this intrinsic similarity, the community structure differed among the sampled sites. Our results indicate this variation could be explained by fluctuation in the soil pH.

Therefore, pH constitutes an important predictor for bacterial rhizospheric community structure in soils under cacao production. This divergence is also reflected in the relative abundances of the bacterial taxa that compose the community.

The functional importance of the analyzed bacteria indicates several strains with biocontrol properties. This, in addition to the biosafety criteria, makes this collection of bacteria, biotechnologically attractive for further analysis.

The unequal distribution of the PGP attributes among the district suggests the potential of these sites as a source of PGPR organisms as well as their functional status.

Although according to our results the pH was the major community-determining factor, the influence of other natural or anthropogenic variables should be analyzed in future studies. This information would represent a complementary set of data for taxonomical and phylogenetical diversity analysis of bacterial communities associated with cacao agroecosystems.

Fundings

This work was financially supported by Programa Nacional de Investigación Científica y estudios Avanzados - PROCIENCIA (Proyecto MICROBIOMA, contrato PE501081028-2022-PROCIENCIA) and the project “Creación e Implementación del Centro de Investigación e Innovación Tecnológica en Cacao de la Universidad Nacional Toribio Rodríguez de Mendoza de Amazonas” (CEIN-CACAO), CUI N° 2315081.

CRediT authorship contribution statement

Jhusephin Sheshira Crisostomo-Panuera: Formal analysis. **Amira Susana del Valle Nieva:** Writing – review & editing, Methodology, Formal analysis. **Manuel Alejandro Ix-Balam:** Writing – review & editing, Visualization. **Jorge Ronny Diaz-Valderrama:** Writing – review & editing. **Eliana Alviarez-Gutierrez:** Writing – review & editing. **Segundo Manuel Oliva-Cruz:** Writing – review & editing, Funding acquisition. **Liz Marjory Cumpa-Velásquez:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Oliva-Cruz, Segundo Manuel reports financial support was provided by National Council of Science Technology and Technology Innovation.

Acknowledgment

The authors are grateful to Ing. Elgar Hernandez-Díaz for supporting the collecting sampling.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e28453.

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