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Comparative study of the rhizosphere microbiome of *Coffea arabica* grown in different countries reveals a small set of prevalent and keystone taxa

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ABSTRACT

Coffee (*Coffea arabica*) is a globally important crop and its cultivation demands the use of agrochemicals and pesticides. Recently, plant-associated microorganisms have emerged as an unexploited resource to enhance crop performance and sustainability. In particular, the rhizosphere microbiome has been identified as a hotspot for plant health, by providing tolerance to biotic and abiotic stresses as well as regulating phenological traits. However, its composition varies widely across crops and environmental conditions. Hence, the knowledge of the "common core" taxa, which are a subset of the microbiome that is persistently associated, across different environmental gradients and conditions, to a host species, represent valuable targets for the development of microbiome-based management approaches. This study aimed to characterize and determine the rhizosphere-inhabiting microbial groups (common core and most prevalent taxa), associated with the *Coffea arabica* plants in different geographical locations. To assess the structure of the coffee rhizosphere microbiome, we performed the 16 S rRNA amplicon analysis of samples collected across six locations spanning four countries and grown under different climate conditions, soil type, agroecosystem management, plant vegetative stage and age. Our findings allowed us to delineate and identify a robust set of six bacterial taxa, namely *Sphingobium*, *Rhizobium* group, *Acidibacter*, *Sphingomonas*, *Burkholderia* group and *Amycolatopsis*, which are likely to play an important role for the growth and health of coffee plants. Using network analysis, we observed that some of the six "common core" taxa were highly interconnected with the resident community, being also keystone taxa of the coffee rhizosphere microbiome. Furthermore, we performed a high-throughput targeted isolation resulting into 162 rhizosphere bacterial strains, some of them related to the most prevalent taxa. These isolates represent a working bacterial culture collection to be used in the future for the development of microbiome-based solutions for a more sustainable coffee farming.

1. Introduction

The rapid improvements in DNA sequencing technologies and data analysis have led to an explosion of data providing insights into the makeup of the bacterial communities associated with a growing number of plant species and crops. These microorganisms inhabit primarily the root environment (rhizosphere and rhizoplane), and to a lesser extent the leaf (phyllosphere), seed (spermosphere), and internal (endosphere) plant environments (Mendes et al., 2013). The rhizosphere represents the main entrance for the root colonizing microbes, which are attracted

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by the nutrients provided by the plant, and are actively recruited from the surrounding soil by the plant host (Zancarini et al., 2021). Several of these microbes harbor mechanisms to promote plant growth, which include phyto-stimulation, nutrient mobilization, antagonism of plant pathogens and tolerance to abiotic stresses (Mayak et al., 2004; Niu et al., 2017). Therefore, management of rhizo-microbiome offers an opportunity to enhance the sustainability of the agroecosystems. However, given the complexity of the natural interactions among bacteria, it is challenging to optimize a microbial application/solution (Schlaeppi and Bulgarelli, 2015). Additionally, plant microbiome composition, diversity and abundance is affected by multiple biotic, abiotic and host-related factors (Bulgarelli et al., 2012; Dastogeer et al., 2020; Trivedi et al., 2020). Despite the large number of influencing factors, different plants often share a "common core" rhizosphere microbiome composed of mainly Proteobacteria, Actinobacteria and Bacteroidetes, indicating that plants greatly shape, influence and tailor the bacterial community composition in their immediate vicinity (Kudjordjie et al., 2019). The enrichment of selected microbial groups (core microbiome), consistently associated with a particular plant under all environmental conditions and gradients, is the result of the co-evolutionary interactions between the plant and its microbiome (Trivedi et al., 2020). The pervasive nature of these microorganisms, suggests that they play important roles in the regulation of host function, performance and health. Consequently, the scientific community is now focusing the attention on the relatively small and impactful "common core microbiome" of economically important plants, in order to provide new knowledge on the ecological role and on the relationships between plants and their associated microbiomes and to design effective microbiome-based solutions to address agronomic challenges (Estendorfer et al., 2020; Simonin et al., 2020; Singh et al., 2020). Along with the concept of "common core" microbiome, Agler et al. (2016) developed the concept of "keystone taxa" to describe the taxa that are closely associated with many other taxa within the microbiome. These connected microbes are important for plant health, as they mediate the interaction between plant and microbiome, by helping in the recruitment of beneficial bacteria or prevent invasion of pathogen (Agler et al., 2016). Therefore focusing on the "common core" and keystone taxa is expected to simplify the analysis of the microbiome, and to identify the key-component of the rhizosphere community and help devise microbe-based solutions for a more sustainable agriculture.

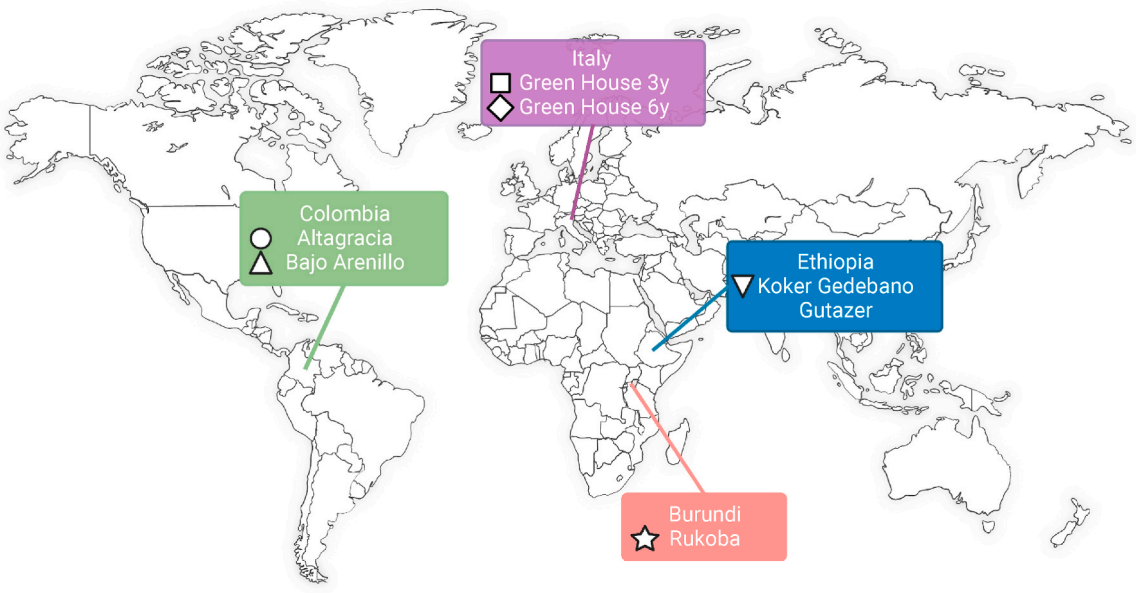
The coffee tree (*Coffea* spp.) is a woody perennial evergreen dicotyledon belonging to the *Rubiaceae* family; it is among the world's most widespread and valuable crops (FAO, 2018). Coffee is the second most consumed beverage after water and the most traded tropical agricultural commodity. The plant can adapt to a range of environmental conditions and agricultural practices, therefore it is one of the main exported products for several developing countries (Senbeta and Denich, 2006; Gole et al., 2008; Mussatto et al., 2011). However, the intensive coffee production systems result in serious environmental issues such as soil degradation, infertility and generation of by-products and wastes. The current knowledge on the microbial community of the coffee plant is rather limited and the practical use of the microbiome data for a more sustainable agriculture is largely unexplored. Indeed, the agricultural practices are moving towards the use of bacteria to enhance plant growth and health, and, in this context, the isolation of plant-specific beneficial strain can be crucial. A few studies have contributed insights into the principal determinants of the coffee microbial communities (both fungi and bacteria) reporting the variation of the coffee endosphere microbiome across climatic gradient and plant age (Fulthorpe et al., 2020; Jurburg et al., 2020), studying effects of the coffee plant genotype on the microbial community structure (de Sousa et al., 2022), describing the leaf-associated microbiome of coffee and its correlation to manganese and calcium (Sousa et al., 2018) and defining the microbiome of coffee beans (Oliveira et al., 2013), and of the coffee leach waste in coffee machines (Vilanova et al., 2015).

In view of the limited knowledge on the *Coffea* rhizosphere microbiome, in the present study we investigated the coffee-associated rhizobacteria community with a focus on the stable and prevalent group of rhizosphere-inhabiting bacteria across different geographical areas. Rhizosphere samples of *Coffea arabica* plants were collected from six different geographical locations spanning four countries in three continents. The plants were grown under different climate conditions, soil type, agroecosystem management, plant vegetative stage and age. Through 16 S rRNA community sequencing we described the bacterial communities associated with the coffee plant roots and provided information on the coffee "common core" and keystone taxa (i.e. the bacteria that are most frequently encountered in the coffee rhizosphere microbiome and are highly interconnected with the total community), their co-occurrence patterns, and indications about the "variable bacteriome" (i.e. location specific). A list of the most stably-associated microbiome members of the coffee crop has been drawn and a set of samples has also been used to isolate, classify and establish a working bacterial culture collection of coffee rhizosphere strains, to be used in the future for developing bacterial solutions for a more sustainable coffee farming.

2. Materials and Methods

2.1. Experimental design and sample collection

A total of 84 rhizosphere samples from *Coffee arabica* plants were collected and analyzed. The samples were taken from four countries spanning three continents, namely Burundi, Colombia, Ethiopia and Italy. Details regarding the number of samples from each location, sampling periods, plant age, developmental stage and management strategies are summarized in Fig. 1. In brief, samples from Colombia were taken from two separate sites (Altagracia Pereira (Risaralda); coordinates: 4.738905 N, −75.706035 W and Bajo Arenillo, Manizales (Caldas); coordinates: 5.053788 N, −75.552489 W), Burundian and Ethiopian samples were taken from one single site, located in Rukoba (coordinates: 3.3986563 N, +29.9076798 W) and Koker Gedebano Gutazer (coordinates:: +8.8883 N, +39.15000 W), respectively, and samples from Italy were collected from two greenhouses located in Udine province (coordinates: 45.88613 N, 13.05718 W). The samples were collected between December 2018 and February 2019, during "early or late fruit" period. Coffee plants at different ages where sampled, ranging from 3 to 25 years (Fig. 1). Plants grown under different management strategies were selected. In particular, coffee trees from Burundi and Ethiopia were all grown in open fields under shed-exposed, while coffee plantations in Colombia were sun-exposed monoculture plots and plants collected in Italy were grown under greenhouse conditions (coffee plants were grown in pod using a commercial substrate and were periodically fertilized through drip irrigation, maintaining temperature minimum 14 °C in winter and natural sun conditions). For each site of sampling, 12–24 biological replicates were randomly selected in order to collect the most distant plants. For each biological replicate, three technical replicates were sampled and merged. To collect the root rhizosphere, the top 5 cm of soil was removed, and fine roots (approximately 1 mm in diameter) from a depth of 10–20 cm were collected. The roots were shaken gently to remove the excess of soil attached to them. Approximately, 2–3 mm of root adhering soil was preserved and each of the tree replicates belonging to the same plant were deposited in a 50-mL falcon tube. Once in the laboratory, 20 mL of 1 x PBS (phosphate saline buffer) was added to the roots and vortex for 5 min to homogenize the sample. Afterwards, the roots samples were subjected to sonication using a sonication bath at low intensity and then the roots were discarded. Tubes containing soil, buffer and rhizosphere fraction were centrifuged for 10 min at +4 °C, 4,000×*g* and the pellet containing the rhizosphere fraction was immediately frozen with liquid nitrogen and stored at −80 °C for the DNA extraction. An aliquot of each sample was treated with PBS and glycerol 20% for future rhizosphere-associated bacteria isolation.



Country	Location	#Sample	Year	State of plantation	Management	Age
Burundi	Rukoba, ISABU	12	December, 2018	early fruitful state	shade-exposed monoculture	18y
Colombia	Altagracia, Pereira (Risaralda)	12	February, 2019	early fruitful state	sun-exposed monoculture	15y
	Bajo Arenillo, Manizales (Caldas)	12				25y
Ethiopia	Koker Gedebano Gutazer	24	December, 2018	late fruitful state	shade-exposed monoculture	18y
Italy	Greenhouse, Udine	12	January, 2019	early fruitful state	greenhouse conditions	3y
	Greenhouse, Udine	12				6y

Fig. 1. Experimental design of the study. Study sites represent four countries and 6 geographical locations, as shown in the world map. Details regarding (i) country of provenience of the samples, (ii) sampling site location, (iii) number of samples collected in each sampling site, (iv) year of the sampling, (v) developmental stage of the coffee plants, (vi) type of management and (vii) age of the plants, are summarized in the table.

2.2. DNA extraction, library preparation and sequencing

The rhizospheric DNA was extracted with the Power Soil DNA isolation kit (Qiagen) following the manufacturer's instructions. The DNA quality and quantity were determined by using a NanoDrop device (Thermo Scientific, Wilmington, DE). The DNA extracted was used to amplify the V3 and V4 hypervariable region of the 16 S rRNA gene using barcoded primers and PCR conditions following Illumina Inc.'s protocol (Illumina Inc., San Diego, CA, USA). Briefly, individual barcoded libraries were directly prepared by PCR using long primers (Klindworth et al., 2013) incorporating the Illumina adapter sequences (16 S_Amplicon_PCR_Fw: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; 16 S_Amplicon_PCR_Rv:GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC), which allow pooling multiple samples into one run of sequencing. Following the first amplification, a cleaning step was performed using the AMPure XP bead clean-up (A63880l; Beckman Coulter Inc., Brea, CA, USA). A second PCR reaction was then performed to attach dual index and Illumina sequencing adapters using the Nextera XT Index Kit; followed by a final AMPure XP bead clean-up. Amplicons size, integrity, and purity were checked using the Bioanalyzer equipment (Agilent Inc., Santa Clara, CA, USA) and the library concentration was measured by fluorimetric quantification using Qubit 2 (Invitrogen Inc., Carlsbad, CA, USA). Finally, libraries sequencing was performed using 2 × 250 bp MiSeq.

2.3. Sequence data processing and bioinformatic analyses

The.fastq files were imported into qiime 2 (Bolyen et al., 2019), the clustering of reads into Amplicon Sequence Variants ASVs was done using the DADA2 plugin (Callahan et al., 2016), and taxonomic assignment was done based on the SILVA database (release 138) (Quast et al., 2012). Diversity values were calculated at a rarefaction depth of 1182 reads, this depth retained over the 35.7% of the ASVs in 73 samples (the 86,90% of all). The dataset was imported in R using the package qiime2R (Bisanz, 2018), and the subsequent analyses and plots were drawn using either phyloseq or microbiome R-packages (McMurdie and Holmes, 2013; Lahti et al., 2017). The prevalence of each taxon (i.e. the number of samples in which the taxon was detected) was determined at the level of ASV and Genus. For the latter, we considered as MPT the genera occurring in more than the 60% of the samples (i.e. more than 50 samples). ASVs that were significantly more abundant in specific locations were determined using maaslin2 (Mallick et al., 2021). To evaluate the similarity among the structures of the microbiomes from different sites we compared the list of genera, ranked by average relative abundance, with the "correspondence-at-top" approach (Irizarry et al., 2005). To infer co-occurrence of bacterial genera among the samples, we exported the table at the genus level, filtered to keep the genera occurring in at least 5 samples, and calculated the SparCC value

(Friedman and Alm, 2012), using the software fastspar (Watts et al., 2019). We filtered out correlations in the range −0.3 - 0.3 and correlations with p-value >0.05. The statistics and the plot of the resulting network were performed using cytoscape (Shannon et al., 2003). Keystone bacterial taxa were identified as in Tipton et al. (2018) and Zwirzitz et al. (2022) (Tipton et al., 2018; Zwirzitz et al., 2022). Briefly, the nodes presenting the highest betweenness centrality and node degree were considered as keystone taxa. The network analysis was performed both on the whole dataset and on the dataset of each location. The metagenome functional content was predicted using picrust2 (Douglas et al., 2020), significant differences among locations were inferred by running the Kruskal-Wallis H-test, *p*-values were corrected using Bonferroni correction to account for multiple comparisons.

2.4. Isolation of culturable rhizosphere - associated bacteria from coffee trees

In order to isolate bacteria belonging to the culturable fraction of the coffee plant microbiome, the rhizospheric fraction stored with 20% glycerol at −80 °C was used for plating different dilutions on four selective solid media. Details regarding the media used are presented in the Supplementary Table ST1. Plates were incubated at 25 °C for 2–5 days and pure independent colonies showing distinct colony morphology were picked and streaked on 1/10 TSA plates to ensure the purity of the culture and then stored at −80 °C in 1/10 TSA and 20% glycerol. Amplification of the 16 S rRNA gene was then performed on these single colonies by using fD1Funi 16 S (5′- AGAGTTTGATCCTGGCTCAG-3′) and rP2Runi 16 S (5′- ACGGCTACCTTGTTAGGACTT-3′) primers to amplify the complete 16 S rRNA gene and gain a more precise taxonomic information of the bacterial isolates. Colony PCR was performed after boiling (10′ at 98 °C) a colony suspension in 50 µl of sterile H_2O. PCR products were purified by using Gel extraction and PCR Clean-Up System purification kit (Euroclone S. p.A). The sequencing performed with primers 907 R (5′- CCGTCAATTCMTTTRAGTTT-3′) and 785 F (5′- GGATTAGATACCCTGGTA-3′) was realized by GATC (Eurofins Genomics Company, Germany) and identification of the isolates was obtained by BLAST analysis at NCBI (http://www.ncbi.nlm.nih.gov). The complete list of the 162 different isolates present in the culture collection and their taxonomic identification is available in the Supplementary Table ST2.

3. Results

3.1. Bacterial diversity and community structure varies across different geographical locations

After quality check and removal of reads classified as chloroplasts and mitochondria, the number of reads ranged 576–11,092 with on average 2911.63 ± 1852.77 per sample.

The alpha diversity, measured using the Shannon index, ranged 3.96–6.77, with an average of 5.33 ± 0.64 and showed a few significant variations among the locations (Fig. 2A). The samples from Colombia – Altagracia displayed a generally higher diversity, whereas samples from Italy – greenhouse at 3 years had the lowest (Fig. 2A).

The analysis of the beta diversity showed that the coffee plants grown under greenhouse conditions hosted a different bacterial community from the ones cultivated in open field, as they form a distinct cluster on the first axes of the NMDS plot (Fig. 2B). On the other hand, the samples collected from the open fields were distributed according to their country of provenience along the second axis; specifically, Colombian and Ethiopian samples formed two partially overlapping groups, whereas samples from Burundi were scattered on the multivariate space (Fig. 2B). By performing the analysis on three axes it was possible to witness that the overlap of the samples from Ethiopia and Colombia was reduced (Supplementary Fig. S1). Thus, we found that the community structure differed mostly geographically, with particularly strong shifts caused by different type of management such as between plants grown under very controlled environment (greenhouse) and the ones grown under seasonal changes.

3.2. Taxonomic composition of differentially abundant taxa and inferred functions

The taxonomic composition of the coffee rhizosphere microbiome was mainly dominated by Proteobacteria of the α- and γ-class, accounting together for the 55.57% ± 5.22, followed by Actinobacteria

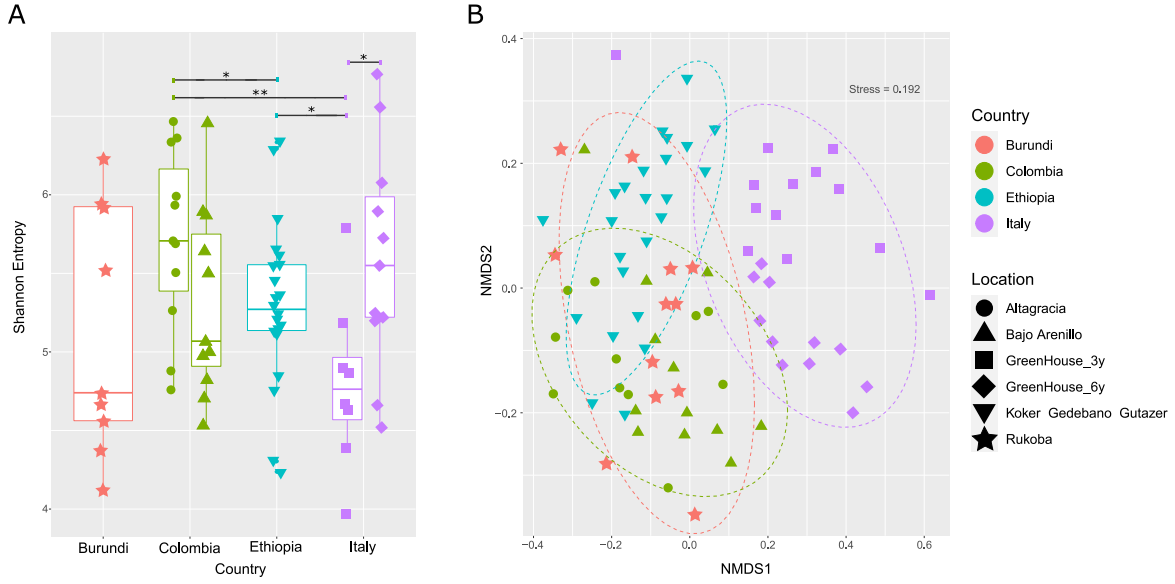


Fig. 2. A) Shannon diversity values, represented as boxplots colored according to the growing country and split between different locations (where applicable). Significant diversity at the Wilcoxon test are defined as × p < 0.05 and **p < 0.01); B) Non-metric multidimensional scaling (NMDS) resulting from the Bray-Curtis distance matrix calculated on the ASVs-by-samples matrix. The dots are colored according to the country of provenience and their shape represents the locations, dashed ellipses show the standard deviation of each group of samples. The Shepard plot displaying the correlation between the true Bray-Curtis distances and the distance on the ordination is in Supplementary Fig. S7.

(12.56% ± 8.01), Bacteroidetes (8.91% ± 3.2), Acidobacteria (7.72% ± 3.4), Chloroflexi (4.65% ± 5.00), Patescibacteria (2.72 ± 1.01), Planctomycetota (2.61% ± 1.75), Verrucomicrobia (2.64% ± 1.57), and other phyla accounting for less than 0.1% (Fig. 3A).

A few taxa were significantly more abundant in some locations (Supplementary Fig. S2), such as *Streptomyces* (phylum Actinobacteria), that was significantly more abundant in the samples from Italy_GreenHouse_3 y; *Chujaibacter* (γ-Proteobacteria) and an unknown species of the family Chitinophagaceae (Bacteroidetes) were significantly more abundant in the samples from Italy_GreenHouse_6 y; *Dyella* (γ-Proteobacteria), *Acidothermus* and *Nocardia* (both Actinobacteria) displayed a higher abundance in samples from Colombia (both locations) and Burundi; an unknown species of the family Ktedonobacteraceae (Chloroflexi) was significantly more abundant in samples from Colombia (both location); *Steroidibacter* (γ-Proteobacteria) and an unknown species of the Acidobacteria Subgroup 2 were significantly more abundant in Colombia – location Altagracia; an unknown species of the family Xanthobacteraceae (α-Proteobacteria) was significantly more abundant in the field samples compared to the greenhouse ones, and the genus *Burkholderia-Paraburkholderia-Caballeronia* (γ-Proteobacteria), here defined as *Burkholderia* group displayed significantly lower abundance in samples from Ethiopia. The inferred functions revealed that samples from Burundi, Colombia and Ethiopia had generally a higher abundance of species with predictable functions compared to the samples from Italy (Supplementary Table ST3). The significantly most abundant functions in samples from Burundi were K01602 - ribulose-bisphosphate carboxylase small chain, K03518 and K03518 - aerobic carbon-monoxide dehydrogenase, K07749 - formyl-CoA transferase, K15868 - bile acid-coenzyme A ligase, K01426 - amidase, K02050 - NitT/TauT family transport system permease protein. The rhizosphere samples from Ethiopia had the following predicted functions that were significantly more abundant compared to the other locations: K06415 - stage V sporulation protein R, K01304 - pyroglutamyl-peptidase,

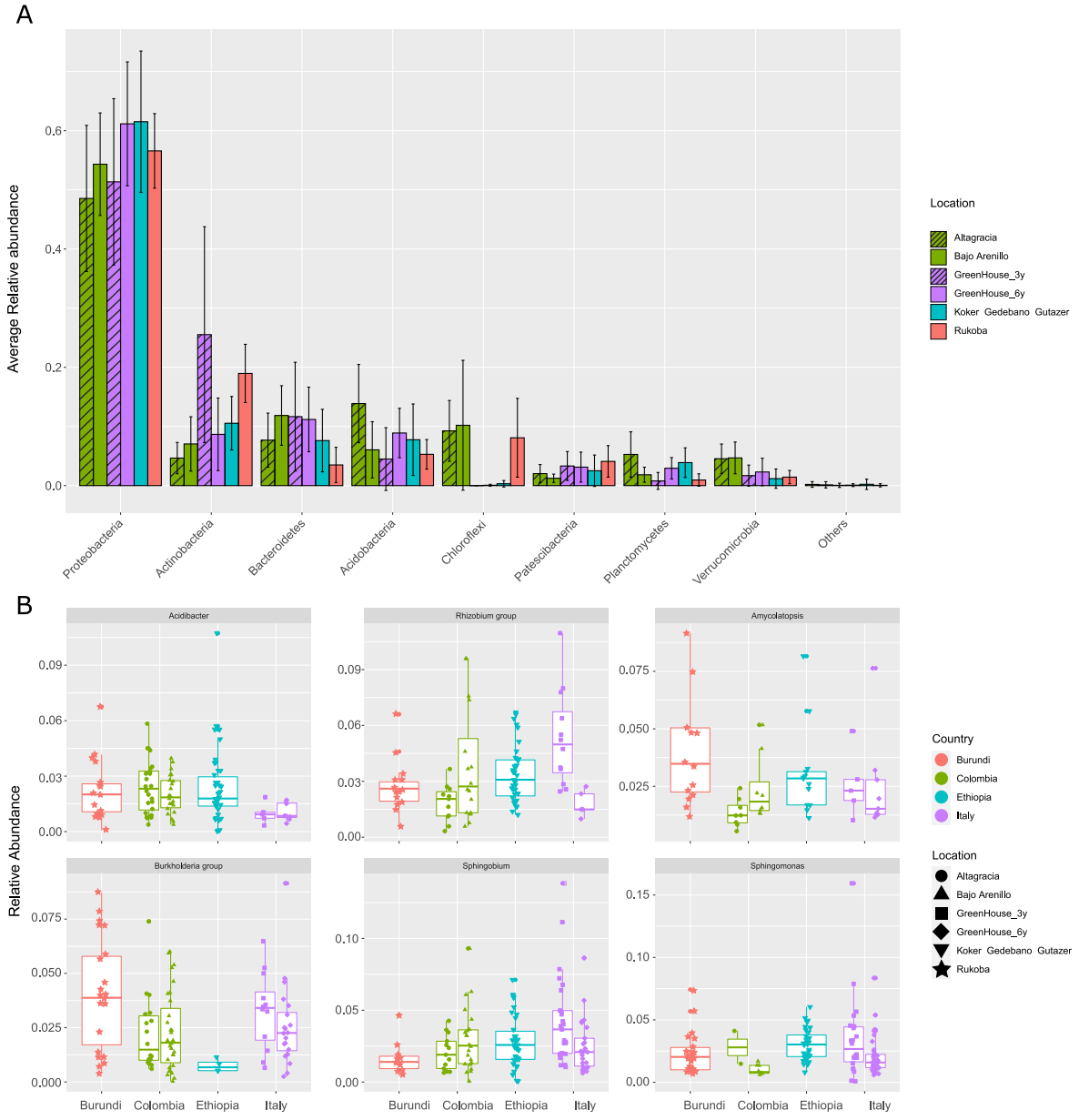


Fig. 3. A) Barchart showing the average relative abundance and standard deviation of each phylum in each location. Bars are colored according to the country and the texture of the bars distinguish the different locations (where applicable); B) Boxplots showing the relative abundance of the genera found in at least the 60% of the samples (that means in >50 samples).

K01974 - rtcA; RNA 3′-terminal phosphate cyclase. The rhizosphere microbiome of the samples grown in Italy had the following functions with significantly higher abundance: K00045 - mannitol 2-dehydrogenase; K03777 - D-lactate dehydrogenase (quinone) and K09992 – uncharacterized protein. No predicted functions were found to be significantly enriched in samples from Colombia.

3.3. A persistent group of rhizosphere-inhabiting taxa presents similar abundance sorting across different locations

In order to define the members of the microbiome most adapted with coffee plant roots, we empirically quantified the coffee most prevalent taxa (MPT) or "common core" microbiome.

At the ASV level, the MPT was present in 32 samples, spanning Burundi, Colombia and Italy, and it was annotated as a member of the Family Xanthobacteraceae (Supplementary Fig. S3). At the genus level it was empirically decided to define as the MPT the genera that were detected in at least in the 60% of the samples and at >0.5% relative abundance. A shortlist of candidate most prevalent taxa included genera such as *Sphingobium* and *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, here referred to as the *Rhizobium* group (α-Proteobacteria, occurring in 67 samples, the 80.72%), *Acidibacter* (γ-Proteobacteria, occurring in 63 samples, the 75.9%), *Sphingomonas* (α-Proteobacteria, in 59 samples, the 71.08%), *Burkholderia* group (b-Proteobacteria 55 samples, the 66.26%) and *Amycolatopsis* (Actinobacteria, occurring in 51 samples, the 61.44%) (Fig. 3B). All those taxa displayed a relatively high average abundance among all locations (Fig. 4A). The rank abundance plots showed that the high abundant fraction of the microbiomes was conserved among the five locations analyzed, with very few species being unique to each environment and representing the "variable – location-specific microbiome". The correspondence between the full ranked lists of bacteria ranged 0.48–0.77, with an average correspondence of 0.62 ± 0.08 (Supplementary Fig. S4). It was visible however that the samples from Burundi displayed high similarities with the samples of Colombia (both Altagracia and Bajo Arenillo) in the high abundant fraction, whereas they displayed high similarities with the Italian samples (both greenhouses) in the low abundance fraction (Fig. 4B).

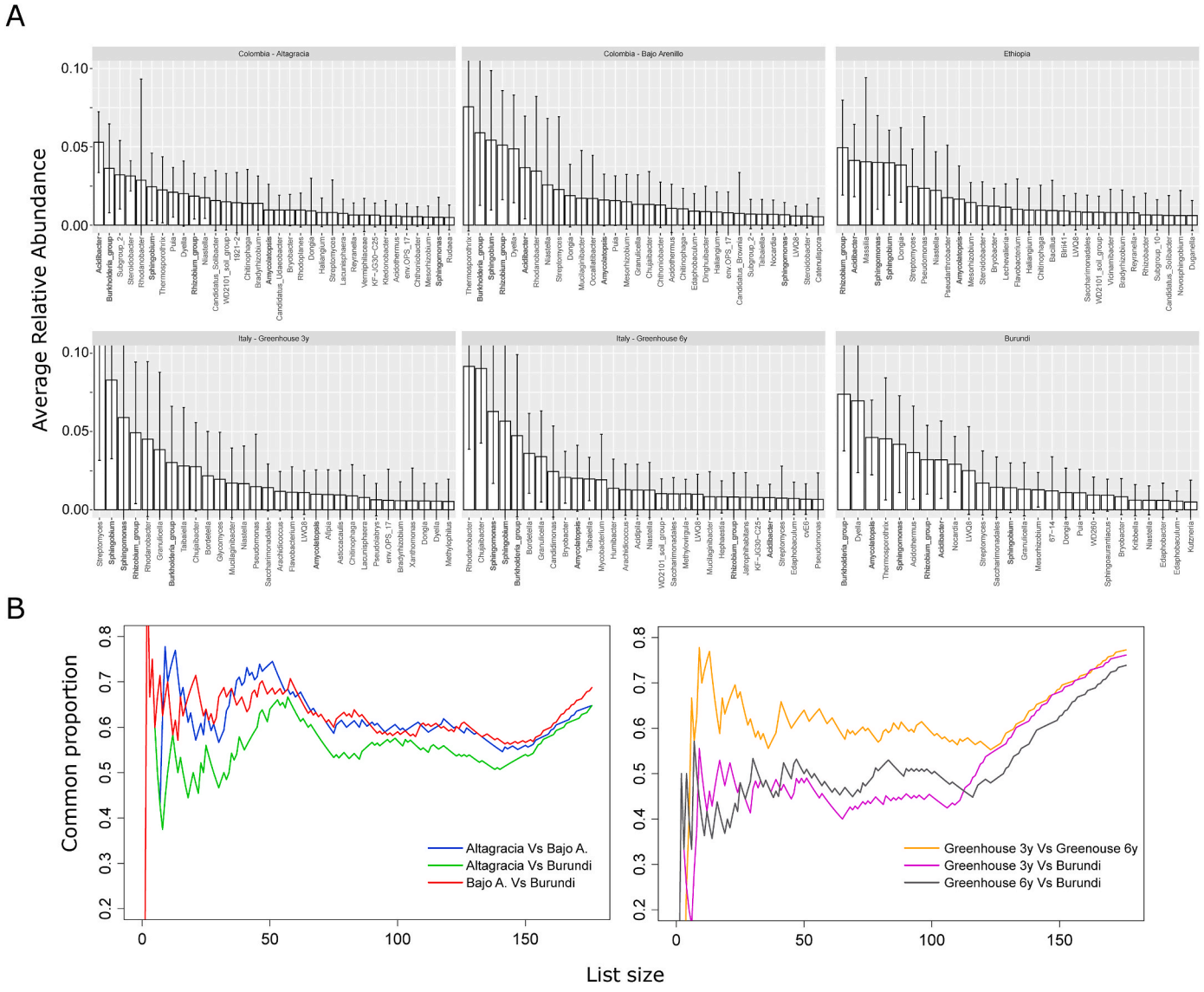


Fig. 4. A) Rank abundance plots showing the genera whose relative abundance was above 0.005, sorted by their abundance in the different locations; B) Selected pairwise comparison of the lists of genera, sorted by abundance, with the "correspondence-at-top" analysis. The six ordered lists in the rank abundance plot of panel A presented roughly the same genera but in different order; the analysis presented evaluates how much each list was similar to each other list (cumulative proportion of 1 would mean perfect identity between two sorting). All-versus-all pairwise comparisons are shown in Supplementary Fig. S4.

3.4. Network analysis reveals strong correlations and keystone taxa belonging to the identified most prevalent taxa of the coffee rhizosphere microbiome

With the aim of identifying possible interactions among the candidate MPT of the coffee microbiome, a co-occurrence network was generated, considering only genera occurring in at least 5 samples, and excluding either the correlations with SparCC values in the range of −0.3 - 0.3 or non-significant values. The resulting network (Fig. 5A) consisted of 90 nodes and 265 edges, there were 204 positive correlation values and 61 negative ones (Supplementary Table ST4). The average number of neighbors was 5.88, the network diameter was 7 with a characteristic path length of 3.25, the network density was 0.06 and the clustering coefficient was 0.4. The majority of the nodes (i.e. the genera) belonged to the Proteobacterial phylum (35), the other phyla represented in the co-occurrence network included Acidobacteria (12 nodes), Actinobacteria (11), Bacteroidetes (10), and 9 more phyla with 8 or less nodes each.

The nodes (i.e. the genera) displaying the highest betweenness values were *Burkholderia* group (betweennes value: 1512.07), *Chujaibacter* (1232.93), an uncultured Acidobacteriales genus (Acidobacteria, 1025.82), *Rhizobium* group (993.63), *Dyella* (781.47), an uncultured Vicinamibacterales (Acidobacteria, 776.31), *Bordetella* (γ-Proteobacteria, 756.65), an uncultured Acidobacterial Subgroup 2 genus (715.88), *Massilia* (γ-Proteobacteria, 682.91) and Thermosporothrix (Chloroflexi, 595.69). Interestingly, the *Rhizobium* group and *Burkholderia* group, which are two out of the six MPT of the coffee microbiome, showed a very high number of interactions with other members of the bacterial community, suggesting likely important functions in shaping and maintain the microbiome. According to the number of degrees that each taxa has in relation to the other members of the microbiome and the value of betweenness, the *Burkholderia* group was detected as keystone taxa, possibly playing some important ecological roles in the coffee microbiome network (Supplementary Fig. S5). Other keystone bacterial genera are *Candidimonas* (γ-Proteobacteria), *Bordetella*, *Chujaibacter* and an unknown species of the family Acidobacteriales (Supplementary Fig. S5). Performing the same analysis at each specific-location level, we observed that the keystone species showed a marked site specificity (Supplementary Table ST5), as very few keystones identified at the global level were also retrieved at the specific-location level. The genus *Bordetella* was the only keystone found in Italy_3 y, whereas the genus *Candidimonas* was found in Italy_6 y, *Dyella* and an uncultured Acidobacterales were found as keystone nodes in the samples of Colombia-Altagracia.

3.5. Isolation and characterization of a set of culturable strains from Coffea arabica rhizosphere microbiome

In order to begin to further study whether the taxa selected as "common core" and most prevalent in the coffee rhizosphere microbiome are impactful for the health and performance of the coffee plants, it was decided to isolate some strains belonging to the shortlist of the candidate core bacterial taxa. A culturable bacteria collection of 162 different strains was generated as described in the Materials and Methods section. Strains isolated also belonged to the *Rhizobium* group, *Burkholderia* group, *Sphingobium* and *Sphingomonas* (Supplementary Table ST2) genera which were assigned as "common core" taxa (see above). Importantly, we were also able to isolate several strains belonging to *Streptomyces* genus, which is not one of the most prevalent taxa of the coffee rhizosphere microbiome across different locations and conditions, but it is the most predominant in the rhizosphere of the samples of coffee plants grown in Italy. We searched for the most similar ASVs among the isolates by comparing the V3–V4 region of the full-length 16 S of the isolates to the sequences of the ASVs, and we found that two isolates classified as *Paraburkholderia* and two isolates classified as *Rhodanobacter* were highly similar (with one nucleotide of difference) to ASV present at low abundance in samples from Italy, Colombia and Burundi. This bacterial collection generated here from *Coffea arabica* rhizosphere will be further used to perform future studies on dynamics and correlations between candidate core bacterial taxa, in order to develop novel microbial probiotic solutions for coffee growth and agriculture.

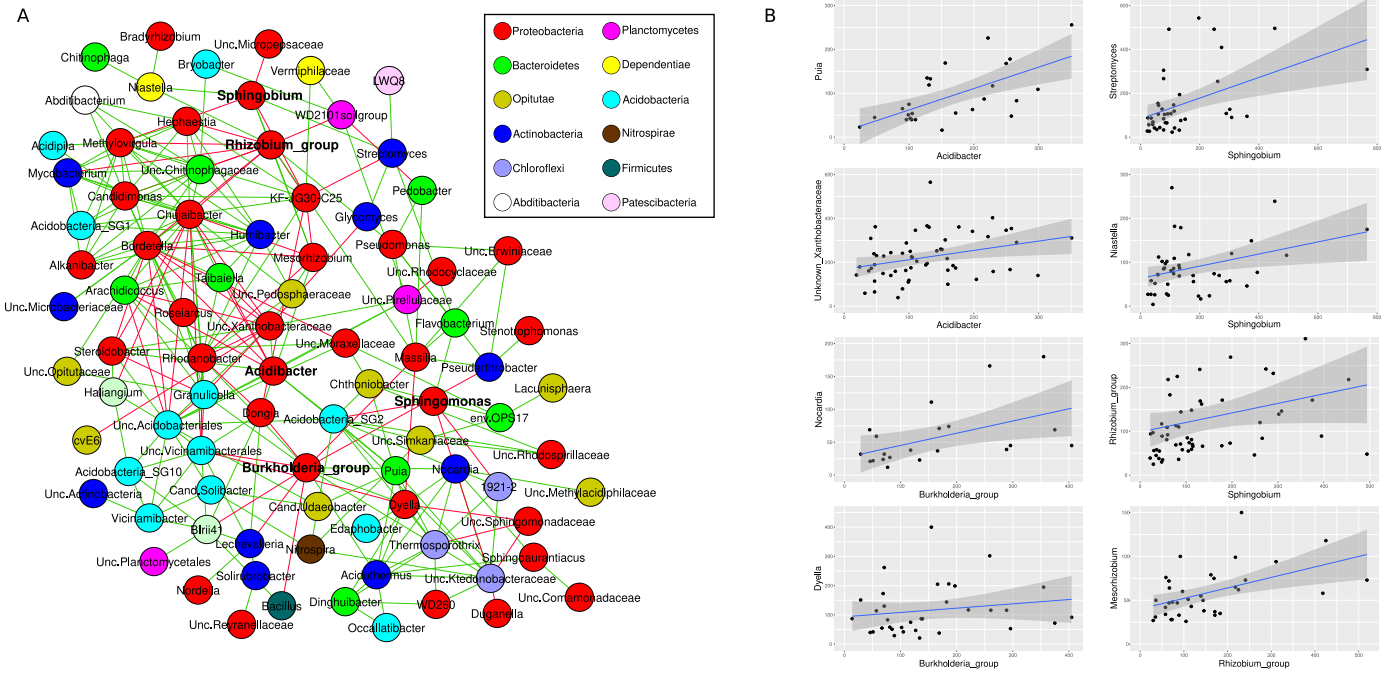


Fig. 5. A) Correlation network inferred using the SparCC value, the nodes are the genera, and they are colored according to their phylum. Only correlation values > |0.3| are shown and the most prevalent genera are in bold. Edges are colored according to the correlation sign (green: positive correlation, red: negative correlation); B) Dotplots displaying the absolute abundance of pairs of species, the regression line (in blue) and the confidence intervals (grey belts) calculated using linear model. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

The translational potential of microbiome studies to support plant health and productivity is still at an early stage; this is in part because it is challenging to establish which member(s) of the microbiota play major roles in the community structure, in plant health and fitness (Arif et al., 2020). In this study, the taxonomic composition of the rhizosphere microbiome of *C. arabica* plants from six different geographical locations spanning four countries was determined in order to identify a conserved group of key members of the microbiome which are likely to contribute to plant fitness. Understanding which are the most prevalent and "common taxa" of the rhizosphere microbiome of each crop is the first step in the development of solutions for more sustainable agricultural systems.

4.1. *Bacterial diversity and richness mostly differ between coffee plants at different growth stages, but not due to geographical location and/or type of cultivation*

This study determined and analyzed the rhizosphere from 84 coffee plants grown under different environmental and plant conditions (such as temperature and rainfall, soil chemical profile, plant developmental stage, plant age and types of management) in order to identify bacteria belonging to the "variable microbiome", which can be putatively influenced by the combination of these components and to delineate a set of common, prevalent and keystone bacteria, which are less influenced by environmental factors. We therefore expected differences between the samples analyzed, as all the above mentioned factors have been associated in changes in the structure and taxonomic composition of plant microbiomes (Dastogeer et al., 2020). However, no dramatic shifts were observed in the relative abundance of the main taxa, suggesting that the selective pressure exerted by coffee plants plays an important role in the rhizosphere microbiome composition and maintenance. This supports the hypothesis that the bacterial community in a healthy situation is featured by biologically significant associations among OTUs which could be either symbiotic or functionally related, and by a high number of bacterial genera which are present in similar amounts (Vandenkoornhuyse et al., 2015). It has also been suggested that the selective pressure on coffee seed size in the Arabica genotype led to a uniform composition of the seed-born microbiome, decreasing the biodiversity (Purugganan and Fuller, 2009). The rhizosphere alpha-diversity was similar between samples from different locations of plants of the same age such as Burundian and Ethiopian ones; however it was evident that the age of the plants significantly altered the microbiome richness. Younger plants (Italy_Greenhouse_3 y) displayed a microbiome with lower diversity compared to the older ones, while coffee plants at 25 years, as the ones from Altagracia in Colombia, presented the highest values of Shannon index. This result is in contrast with the study of Jurburg et al. (2020) that found that older plants had microbiome with lower alpha-diversity; a possible explanation could be that the combination of several factors influence in concert the biodiversity. Previous studies suggested that rhizosphere microbiome composition may vary between plants at different developmental stages (Chaparro et al., 2014); one possible reason is that root exudates present different chemical compounds at different developmental stages (Pantigoso et al., 2020) hence different bacteria are selected and/or maintained. It can be speculated further that different ages are characterized by different specific needs under the nutritional point of view, and therefore the concentration of certain minerals, such as potassium and calcium, changes between coffee plants at different stages of growth. However, it could not be ruled out that the soil microbiome may be one of the reasons for the difference in the coffee rhizo-microbiome composition. It was hypothesized that plants cultivated in the greenhouse, probably have a less diverse microbiome compared to the one grown in open field which are constantly subjected to seasonal changes and varying environmental factors. However, in our samples, we only detected a significant difference with the plants at 3 years, hence the greenhouse condition did not strongly impact on the community richness.

4.2. *Coffea arabica microbiome composition is mainly driven by the type of cultivation and geographical location*

The beta-diversity analysis revealed that the composition of the microbial communities was mainly driven by the type of coffee cultivation, followed by the geographical location. In particular, the plants grown in the Italian greenhouse, clustered separately from the ones grown in open fields, suggesting that the type of management significantly influences the composition and structure of the microbiota. In fact, differences in soil carbon, phosphorus, and nitrogen are strong determinants of bacterial community composition (Delgado-Baquerizo et al., 2017) and may explain why management affect the microbiomes. Additionally, a clear clustering was observed due to the geographical locations, suggesting that factors like temperature, rainfall and soil-type account for the rhizosphere microbiome composition, as already demonstrated in other studies (Qu et al., 2020). Interestingly, the samples from Burundi are the most scattered in the ordination plot, indicating that the rhizospheres of these plants display very heterogeneous composition and structure. A possible explanation is that precipitation significantly impacts the structure of the rhizosphere microbiome, especially in the relative abundance of specific taxa, as several plant compartments have been demonstrated to be influenced by rain events (Aslam et al., 2016; Mavrodi et al., 2018). In fact, the samples from Burundi were the only ones collected during the rainfall season. The high abundant fraction of the microbiome was very similar in all locations; however, some locations were more alike than others. Samples from Burundi featured genera in the high abundance fraction (the first 50 ranks in Fig. 4B) that showed high similarity in their sorting with the ones in Colombia, whereas the genera in the low abundance fraction, were similar to the Italian samples. The similarities in the ranked abundances of specific taxa could be associated to similar ecological functions.

4.3. *Defining the "common core" microbiome of Coffea arabica across multiple conditions*

Deciphering the "common-core microbiome" evidenced that *Amycolatopsis* was one of the most prevalent genera of the coffee rhizosphere microbiome; *Amycolatopsis* is closely related to the *Nocardia* genus (which is also quite prevalent). Many species of the phyla Actinobacteria play an important ecological role in material degradation, as they can break down difficult-to-decompose organic compounds such as methanol, keratin and aromatic hydrocarbons. Some species are extremely heavy-metal-tolerant to nickel or copper (Song et al., 2021). Members of Actinobacteria have also been identified as a dominant component of the coffee microbiome in other studies (Fulthorpe et al., 2020; de Sousa et al., 2022), indicating that it is an important member of the coffee microbiome of the rhizosphere. The prevalence of Actinobacteria, especially *Amycolatopsis* in Burundi, Colombia and Ethiopia and *Streptomyces* in Italy, is an interesting finding because these are generally underrepresented in the rhizosphere of other plants (Peiffer et al., 2013; Jiang et al., 2017; Stopnisek and Shade, 2021). These two bacterial genera are known to produce high levels of secondary metabolites such as antimicrobial compounds in the rhizosphere (Cordovez et al., 2015). This indicates a likely intimate relationship with the plant in the coffee rhizosphere which points towards the possible use of strains of *Amycolatopsis* or *Streptomyces* as coffee probiotic bacteria. Coffee is a high-nutrient-demanding-crop, especially potassium (K) and nitrogen (N), which are needed by the fruits (Caldwell et al., 2015) and hence they are acquired by the plant from the soil with the aid of rhizosphere bacteria. Previous studies demonstrated that the rates of N-compounds in the rhizosphere soil of *C. arabica* were higher compared to bulk soil,

this fact is most probably linked to the release of enzymes, H+ ions, and carbohydrates in the root zone and to the activity of ammonia-oxidizing bacteria (AOB), ultimately affecting N availability in the rhizosphere (Munroe et al., 2015). Bacteria belonging to the *Rhizobium* group indeed are found as prevalent in the majority of the samples analyzed, and they may contribute to N availability for the coffee plant. *Rhizobium* itself is well known for being a symbiotic nitrogen fixer, especially in the legumes roots-nodule; not known if they play a role here as free-living nitrogen fixers. The persistent presence of *Rhizobium* strains in the coffee rhizosphere microbiome is particularly interesting and could be associated to its ability to improve the uptake of other macro-nutrients (such as K,P and Mg) for the plant host fitness (Nyoki and Ndakidemi, 2018). Several of the main coffee growing regions are characterized by acidic soils, which are poor in bases and present high saturation of H^+ and Manganese (Mn). Under these conditions, acidophilic bacteria such as strains from *Acidibacter* genus are enriched. This genus contains species which are extremophiles capable of reducing ferric iron, and have been identified as part of the "common-core microbiome" of coffee, suggesting that they can play important functions, such as iron (Fe) acquisition.

Sphingobium and *Sphingomonas* genera have also been identified as prevalent taxa of the coffee microbiome; interestingly, strains belonging to these genera have been reported to promote plant growth by secreting gibberellins and modulating the levels of stress-induced ethylene via the production of 1-aminocyclopropane-1-carboxylic acid deaminase (Shi et al., 2022).

4.4. *Network analysis revealed the Coffea arabica keystone taxa evidencing that some "common core" taxa are also highly interconnected with the total community*

According to the network analysis, *Burkholderia*, *Candidimonas*, *Chujaibacter* and *Bordetella* are likely to act as keystone taxa, since they establish several positive interactions with many species and may play an important role in mediating microbiome responses to the abiotic or biotic stresses (Supplementary Fig. S5). The genus *Bordetella* established the highest number of positive interactions and is well studied for its pathogenic species (i.e. *B. pertussis*) however, despite its widespread distribution and heterogeneity of environmental niches (Hamidou Soumana et al., 2017), the role of *Bordetella* species in plant root microbiome is currently unknown. Similarly, no information is currently available on the ecological role of *Chujaibacter,* which has been found to have the highest betweenness and number of interactions with other bacterial species thus having a role in the structure of the coffee root microbiome (Supplementary Fig. S5). The same analysis done on the total dataset was performed also for each single location, in order to evaluate whether the taxa detected as keystones were identified at each local level. However, the stringent parameters for correlation inference (co-occurrence in at least 5 samples and a SparCC value of at least $|0.3|$) resulted in a very small network for most of the locations. As a consequence, the keystone taxa at each location were not the same, and many of the detected keystones at the global level were not detected locally. Some positive correlations and interactions were also mapped between the most prevalent members of the coffee microbiome; in particular strains of the *Sphingobium* and *Streptomyces* genus, as well as members of the *Sphingobium* and *Rhizobium* group, suggesting likely cooperation. Along with the co-occurrence analysis on the entire dataset, we carried out the co-occurrence analysis stratifying the samples according to the localities, in order to identify location-specific bacterial signatures. We found that most of the patterns found in the total dataset were confirmed also at a local scale (i.e. positive correlations between two species, were occurring more or less strongly in each location). Two remarkable examples of correlation between two genera at a local scale occurred between the genera *Dyella* with *Thermosporothrix* in Burundi and *Sphingobium* with *Niastella* in the greenhouse 6 year samples in Italy. Both of them display a positive value considering all samples, however, in those specific locations the correlation becomes almost linear (Supplementary Fig. S6).

5. Conclusions

In conclusion, the analysis of several rhizosphere bacterial communities belonging to coffee plants grown under largely different environmental conditions, allowed us to delineate and identify a robust set of six bacterial taxa, which are likely to play an important role for the growth and health of the coffee plant. This result is further supported by the identification of positive correlations and possible interactions between some of them. We are currently collecting rhizosphere samples of *C. arabica* in Vietnam and initial data on the microbiota composition is corroborating the observation made here (data not shown). However, to justify a focus of research efforts on this small but frequent encountered subset of taxa, it is critical to experimentally verify these associations and the ability of these key-bacteria to behave as plant-growth promoting bacteria. The targeted isolation of bacterial strains performed in this study is the starting point to develop tailored microbiome-based solutions for coffee crop agriculture. The future confirmation of the positive dynamics among the members of the microbiome will promote the development of a rationally designed microbiome technology for sustainable coffee crop agriculture.

6. Availability of data and materials

DNA sequences can be found under BioProject ID PRJNA868599.

Declaration of competing interest

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

Data availability

I have shared the link to my data

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rhisph.2022.100652.

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