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Microbiota Modulation in Blueberry Rhizosphere by Biocontrol Bacteria

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Abstract: Microbial interactions in agricultural soils can play important roles in the control of soil-borne phytopathogenic diseases. Yields from blueberry plantations from southern Spain have been impacted by the pathogenic fungus, *Macrophomina phaseolina*. The use of chemical fungicides has been the common method for preventing fungal infections, but due to their high environmental impact, legislation is increasingly restricting its use. Biocontrol alternatives based on the use of microorganisms is becoming increasingly important. Using the metabarcoding technique, fungi and bacteria were characterized (via 16S and ITS regions, respectively) from rhizosphere soils of healthy and dead blueberry plants infected by *M. phaseolina*, and which had undergone three different treatments: two biocontrol strategies—one of them a mix of *Pseudomonas aeruginosa* and *Bacillus velezensis* and the other one with *Bacillus amyloliquefaciens*—and a third treatment consisting of the application of a nutrient solution. The treatments produced changes in the bacterial microbiota and, to a lesser extent, in the fungi. The abundance of *Fusarium* was correlated with dead plants, likely favoring the infection by *M. phaseolina*. The presence of other microorganisms in the soil, such as the fungi *Archaeorhizomyces* or the bacteria *Actinospica*, were correlated with healthy plants and could promote their survival. The different genera detected between dead and healthy plants opens the possibility of studying new targets that can act against infection and identify potential microorganisms that can be used in biocontrol strategies.

Keywords: metabarcoding; biocontrol; blueberry; *Macrophomina*; *Fusarium*

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1. Introduction

Blueberry (*Vaccinium corymbosum*) is one of the most consumed berries around the world, with increasing trends in production and sales [1]. Spain is ranked the third-highest country worldwide in terms of blueberry production, with 50,000 tons of berries—with production concentrated in the southwest of its territory [2].

Stem blight is a major disease in blueberries. This fungal disease leads to the fast degradation of the vascular system, thereby causing foliage death and brown discoloration of internal vascular stem tissues, which eventually provoke plant death [3,4]. It is produced by fungi, principally members of the *Botryosphaeriacerae* family, and can lead to plant death [5]; there is ample biogeographic variability. For example, in Florida, *Lasiodiplodia theobromae* and *Neofusicoccum ribisson* are the main fungi responsible for stem blight [6], meanwhile in Chile, *N. arburi* and *N. parvum* have the greatest impact [7]. In the past, *N. parvum*, *N. australe*, *L. theobromae*, and *N. clavispora* were considered the major infection-causing fungi pathogens in Huelva [7]. However, between 2015 and 2017, the phytopathogenic fungus, *Macrophomina phaseolina*—the principal cause of strawberry crop losses—was also reported [3,8]. This fungus is plastic and has a wide range of targets [9]. Furthermore, *M. phaseolina* can persist in soil for many months by adopting resistance forms called sclerotia [10].

M. phaseolina is an anamorphic ascomycete belonging to the *Botryosphaeriaceae* family and is the cause of the stem blight and charcoal rot in red fruits, as well as other illnesses in a diversity of plants [11]. It is an endophytic fungus that enters the plant by using two methods, one physical and the other mechanical. Through the mechanical method, it enters the plant by exerting pressure on the roots that are proximal to the growing germ tubes. For the chemical method, the fungus dissolves the cell wall through the secretion of toxins (botryodiplodin toxin) and enzymes (cellulases and polyglacturonase) [10–12]. The infection can be favored by biotic and abiotic stresses, as well as high temperatures (29–35 °C) [13], hydric stress [14], damage to the cell wall, or the presence of nematodes [15] and other fungi, such as *Fusarium* [16].

There are chemical methods that can prevent the infection caused by *M. phaseolina*. In strawberries, for example, treating the soil with methyl bromide or Dazomet prior to cultivation have been a common practice. Both were recently banned due to their high environmental impact [17,18]. Other synthetic phytochemicals, such as 1,3-dichloropropene:chloropicrin, are being used, but legislation is becoming stricter in Europe, and a ban is probable [19]. Therefore, farmers are increasingly demanding alternative environmentally friendly methods to combat this as well as other pathogens. Disinfection by biosolarization (a technique that combines biofumigation with soil solarization), the use of plastics covers, the selection of pathogen-resistant plants, or the use of microorganisms for biocontrol are starting to be used [20]. Bacteria with PGP (Plant Growth Promoting) activities promote plant growth through various mechanisms, such as iron and phosphorus solubilisation, nitrogen fixation, or phytohormone production. Some of these can act directly or indirectly against crop pathogens. When acting directly, they can secrete antibiotics or produce metabolites that suppress other metabolites, such as siderophores, hydrolytic enzymes, or hydrogen cyanide. When acting indirectly, they can activate the plant's molecular defense mechanisms (induced systemic resistance), thereby triggering the plant's resistance to infection [21]. Previous studies have shown the efficacy of using this type of bacteria against *M. phaseolina* in strawberries crops [22,23].

Most fungal phytopathogenic diseases affecting berries are soil-borne. The persistence of resistance latent structures in the soil can boost these fungal diseases, while the native microbiota in agricultural soils can have a suppressive effect [24–26]. Thus, studying the soil's microbiota from an agriculture perspective is becoming increasingly important [27]. The traditional way of characterizing microbiota is through the culture-isolation of strains from soil samples in the lab. This technique does not provide a full spectrum of the microbial diversity in the soil since many microorganisms cannot be cultured under laboratory conditions. The advent of NGS (Next Generation Sequencing) allows for the characterization of the microbial diversity from complex soil samples [28]. This fact has led to great advances in the study of plant-associated microbiomes [29]. Specifically, the "metabarcoding" technique permits the characterization of biological communities through a gene region that serves as a "barcode". For the soil microbiota, the Internal Transcribed Spacer (ITS) intergenic region is often used for fungi and the 16S rRNA coding region of the ribosome is used for bacteria [30]. The taxonomic classification of these sequences against curated databases has allowed for the taxonomic characterization of the samples [31].

The aim of this work was to study the changes in the microbial community of rhizospheric soils in blueberries suffering from infection—and death—by *M. phaseolina* compared to healthy plants. We also evaluated the effect of three proposed environmentally friendly prevention strategies for fungi disease control on the soil microbiota; two of them were based on bacteria-mediated biocontrol, and the third one was based on a nutrient solution. We expect to find differences in the microbial composition of the rhizosphere of healthy and dead plants, including a higher proportion of *M. phaseolina* in the dead ones. As for the treatments, we expect that they will modify the soil microbiota by decreasing the presence of pathogens and increasing the microorganisms with PGP and pathogen antagonistic properties.

2. Materials and Methods

2.1. Treatments

Trials were carried out at the IFAPA institute experimental farm, El Cebollar—which is located at Moguer (Huelva, 37°14′25.4″ N 6°48′09.2″ W)—in two 50 m long high plastic tunnels with 2 beds each. Three soil treatments based on environmentally friendly strategies for controlling fungal disease were assayed: the first consisted of two bacteria belonging to the IFAPA collection, which had shown in vitro inhibition of *M. phaseolina* growth (A), the second contained a commercial strain of *Bacillus amyloliquefaciens* (B), and the third one contained a nutrient solution (C). We also included a control plot. Each treatment was tested on four beds inside two different high plastic tunnels. Each of the beds was divided into six sections of equal length. The end sections were not planted since they were least homogeneous in terms of irrigation and exposure to climatic conditions. The treatments were applied to the remaining four sections.

Treatment A consisted of bacterial strains AC17 and ACH16, which correspond to *Pseudomonas aeruginosa* and *Bacillus velezensis*, respectively. Both strains had been isolated from blueberry rhizospheric soil and shown to inhibit the growth of *M. phaseolina* in vitro [32]. Treatment B was a commercial biological fungicide containing *Bacillus amyloliquefaciens*. This product had been shown to be effective against a wide range of both biotrophic and necrotrophic pathogens [33]. Treatment C was a commercial nutrient solution containing nitrogen, phosphate, potassium, and amino acids. These minerals promote plant development, and the amino acids should stimulate the formation of new absorbing hairs, thereby enhancing the assimilation of water and minerals. The treatments were applied every 21 days by drip irrigation. In treatments A and B, 2×10^{10} cfu were applied to each plant, and C was treated as established in the technical data sheet of the product. The bacterial application started on 2 July 2020—after pruning—and was repeated 4 more times until 24 September 2020 with the following application dates: 2 July, 23 July, 13 August, 3 September, and 24 September. At that time, application was ceased for harvesting.

2.2. Sampling

A stratified sampling was carried out in February 2021 by taking soil samples that were adjacent to the blueberry plants in the bed. In each treatment, we selected a dead blueberry plant affected by *M. phaseolina* and a healthy plant. The samples were taken with an auger with a 4 cm diameter and up to a 20 cm depth at 4 different points in the vicinity of the plant within the section corresponding to the treatment in question, and then they were poured into a plastic bag and homogenized by manual shaking. The samples were stored and transported at 4 °C to the laboratory where they were frozen at −80 °C until handling.

2.3. DNA Extraction

Prior to DNA extraction, samples were freeze-dried in the Telstar® LyoQuest freeze-dryer. Lyophilisation took place in 2–15 mL tubes with a perforated parafilm covering the mouth of the tube for a minimum of 8 hr at 0.1 mbar at −80 °C, which ensured the complete dehydration of the sample. Dehydration paralyzes all microbial activity and ensures that the microbiota remains unchanged during processing [34], and standardises the subsequent DNA content to a comparable dry weight between samples.

DNA was then extracted from approximately 0.25 g of freeze-dried soil using the Qiagen® DNeasy PowerSoil kit according to the manufacturer's instructions. The negative controls were included in each extraction batch of 13 soil samples—in addition to 2 technical replicates. We included a positive control (ZymoBIOMICS™ Microbial Community DNA Standard II, Log Distribution, #D6311) consisting of genomic DNA from a known mock community with decreasing concentrations of different bacterial strains and two fungi, *Cryptococcus neoformans* and *Saccharomyces cerevisiae*. All soil samples were inoculated with a known amount of an internal control of bacteria and fungi known as spike-in. For bacteria, a commercial control (ZymoBIOMICS™ Spike-in Control I # D6320) containing

the halophilic bacteria, *Imtechella halotolerans* and *Allobacillus halotolerans*, was used. The *Yarrowia lipolytica* strain, CECT 1240, was chosen as the fungus, which was grown in our laboratory at high concentrations and quantified in Petri dishes. The internal control and the mock community allowed us to validate the downstream methodological flow and the sensitivity of the method. The amount of extracted DNA was determined using a UV spectrophotometer (NanoDrop2000 ThermoFisher).

2.4. DNA Metabarcoding Library Preparation and Sequencing of Samples

The genetic characterization by metabarcoding of the bacterial and fungal communities was carried out based on target amplicon libraries that were sequenced in Illumina. For bacteria, primers 515-F [35] and 806-P [36], which are specific to the V3-V4 region of bacterial 16S rRNA—and were recommended in the Earth Microbiome Project protocol [37]—were used, thereby generating a product of about 292 bp. For fungi, primers ITS3 and ITS4, which are specific to the ITS2 regions of the fungal genome and produce an amplicon of about 330 bp [38], were used (Table 1). All primers contained Illumina tails at their 3′ ends that were complementary to the Illumina adaptors, which were added in a second PCR.

Table 1. Locus-specific primer sequences.

	Primers	SEQUENCE (5′–3′)
Bacterial 16S	515F-Y	GTGYCAGCMGCCGCGGTAA
	806R	GGACTACNVGGGTWTCTAAT
Fungal ITS2	ITS3	GCATCGATGAAGAACGCAGC
	ITS4R	TCCTCCGCTTATTGATATGC

PCRs were carried out at a final volume of 12.5 µL containing 4–20 ng of DNA, 0.5 µM of oligonucleotides, 6.25 µL of Supreme NZYTaq 2x Green master mix (NYZTech), and ultrapure water. The PCR reaction consisted of a preliminary denaturation step at 95 °C for 5 min, followed by 25 cycles of a denaturation step at 95 °C for 30 s, annealing at 46/50 °C (16S/ITS, respectively) for 30 s, and then elongation at 72 °C for 45 s, with a final extension for 7 min at 72 °C. A negative control with the reaction reagents and no DNA was included in all PCR reactions. Libraries were run on a 2% agarose gel with GreenSafe (NYZTech) and visualised under UV light.

The oligonucleotide indices required in the multiplexed libraries were added in a second PCR with the same PCR conditions as above, but with 5 cycles and an annealing temperature of 60 °C. Library preparation was carried out following the process described by Vierna et al. [39]. They were purified using Mag-Bind RXNPure Plus magnetic beads (Omega Biotek) by following the supplier's instructions. The libraries were combined in equimolar concentrations following Qubit dsDNAHS assay quantification (ThermoFisher Scientific) and sequenced on a fraction of an Illumina NovaSeq PE250 lane by AllGenetics & Biology SL (La Coruña, Spain).

2.5. Bioinformatics Analysis

The primers that were used for library preparation and sequences with unidentified nucleotides ("N") were removed with *Cutadapt 3.6.9* [40]. The rest of the sequence processing was carried out in *R 4.1.2* [41] using *dada2* [42], with some modifications to accommodate the quality encoding of the NovaSeq system [43]. The sequences were classified into ASVs (Amplicon Sequence Variants), thereby treating each variant individually, as opposed to the traditional OTU (Operational Taxonomic Unit) classification system in which sequences within a similarity threshold (often 97%) are clustered together. The OTU-related system ignores the variation that can occur from just a single nucleotide between close taxa (within genus or species). ASVs provide higher resolution, sensitivity, specificity, and reproducibility [44]. The SILVA database for bacteria [45] and UNITE for fungi [46] were used for taxonomic classification.

For the rest of the analysis, the R package, *phyloseq* [47], was used. The internal controls (spike-in and ZymoResearch mock community), ASVs that were not classified at the phy-

lum level, low prevalence (presence in only one sample) with the *phyloseq_filter_prevalence* function of the *MetagMisc* package [48], and low relative abundance (less than 1.5×10^{-6} in bacteria and less than 4×10^{-6} in fungi) were removed. In addition, sequences that corresponded to eukaryotic sequences (chloroplasts and mitochondria) and contaminations present in controls were removed with the *isContaminant* function of the *decontam* package [49].

2.6. Alpha and Beta Diversity

For statistical analyses, R was used. The alpha diversity was estimated with the observed diversity (number of different ASVs) and Shannon and Simpson index using the *estimate_richness* function and plotted with *plot_richness* from the *phyloseq* package [50]. The observed diversity only considers the number of different taxa in the samples, and the other two indices also measure the evenness and dominance [51]. The relationship between alpha diversity and the different treatments was tested with an ANOVA test using the *stat* package of R. Subsequently, pairwise comparisons were made with Tukey's test. The relationship between the alpha diversity and plant condition (healthy/dead plant) was determined with the generalised linear model from the *lme4* package, assuming that the data follow a binomial distribution (healthy = 1; death = 0) [52].

To evaluate the effect of the treatments and plant condition on the microbiota structure, the beta diversity was explored based on the Bray–Curtis distance [53]. Then, it was visualized in a Principal Coordinate Analysis (PCoA) that was run with the *ordinate* function in the *phyloseq* package. A PERMANOVA (Permutational Analysis of Variance) test using the *adonis* function of the *vegan* package allowed us to partition the variance of the observed dissimilarity between the treatments and plant condition, and test its significance after 999 permutations [54].

To characterize the relationship between the treatments and plant death/survival in the presence of specific genera, the *DESeq* package was used. This package calculates the normalised change in abundance (fold change) and the associated statistical significance (*p*-value). To this end, we contrasted the differences between the treatments and plant condition. The negative fold change values indicated a higher presence of the genus in dead plants or control soils and the positive values indicated a higher presence in healthy plants and after a given treatment. DESeq performed an internal normalisation of the data in which it detected the possible outliers and reduced the dispersion of the data, which provided stability to the model [55].

For relative abundance plots, the *microbiome* package was used [56], whereas *ggplot2* was chosen for the other representations [57].

3. Results

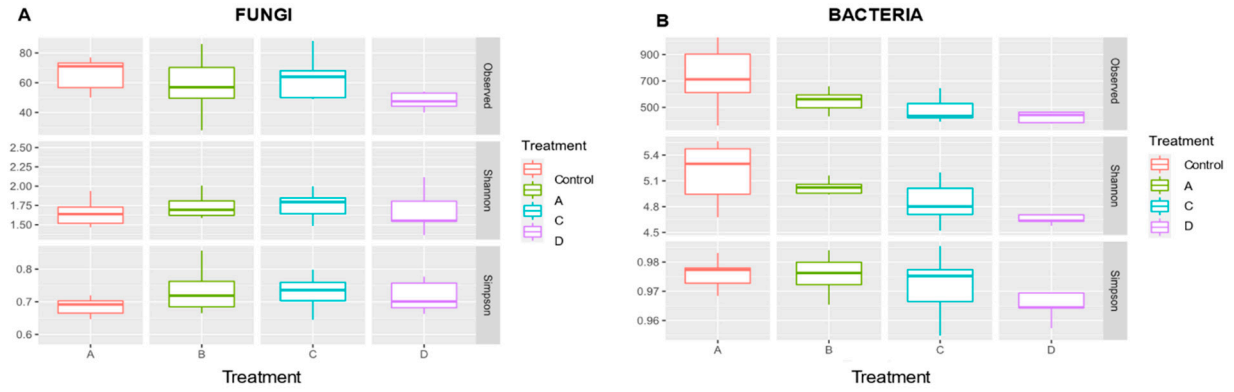
A total of 2,436,437 fungal sequence reads were produced, which yielded 237 ASVs and detected 91 genera across all samples. For bacteria, 2,748,369 reads were analyzed, identifying 2291 ASVs and 215 different genera.

3.1. Sensitivity of the Method

The detection of the microorganisms of the mock community allowed us to establish the sensitivity of the method. In the case of bacteria, *Lactobacillus fermentum* was detected, but not those bacteria found in lower concentrations, which means that the detection limit for bacteria was at least 0.012%. In the case of fungi, *Cryptococcus neoformans* was detected, so the sensitivity for fungi is at least 0.0014%.

3.2. Alpha Diversity

The observed diversity for fungi was low (around 50–70) (Figure 1A). The one-way ANOVA test for the comparison of the effect of the treatments on the alpha diversity pointed to non-significant differences ($F(3,27) = 1.931$, $p = 0.267$).



Treatment	Mean Observed	Mean Shannon	Mean Simpson
Control	65.43 ±10.75	1.64 ±0.18	0.68 ±0.06
A	58.38 ±18.12	1.81 ±0.32	0.73 ±0.07
B	63.13 ±12.40	1.77 ±0.16	0.74 ±0.04
C	51.83 ±13.72	1.67 ±0.28	0.72 ±0.05

Treatment	Mean Observed	Mean Shannon	Mean Simpson
Control	735.86 ±237.16	5.07 ±0.40	0.97 ±0.01
A	549.63 ±75.55	4.85 ±0.17	0.97±0.01
B	480.75 ±90.68	4.69 ±0.24	0.97 ±0.01
C	428.20 ±43.83	4.55 ±0.12	0.96±0.01

Figure 1. Alpha diversity. Graphic representation of the alpha diversity as a box plot and values in tables (median with 0.25 and 0.75 quantiles). (**A**) Observed diversity and the Shannon and Simpson indices for fungi. (**B**) Observed diversity and the Shannon and Simpson indices for bacteria.

All diversity indices had higher values for bacteria than for fungi (Figure 1B). In all cases, the values close to unity for the Simpson's diversity indicate dominance within the community. The observed and Shannon's diversity indicate a higher diversity in the control and for treatment A (*P. aeruginosa* + *B. velezensis*) than in the other two treatments. Furthermore, the differences in diversity between healthy and dead plants were studied, and the Shannon diversity was observed to be 0.16 points higher in healthy plants (PrChisq = 0.066). The one-way ANOVA used to study the relationship between the alpha diversity and the treatments revealed a significant effect ($F(3,24) = 6.41$, $p = 0.002$). Tukey's HSD test revealed that the alpha diversity differed between treatments B (*B. amyloliquefaciens*) and C (nutrient solution) with respect to the control ($P_{B\text{-control}} = 0.006$ and $P_{C\text{-control}} = 0.004$). There were differences between the alpha diversity and the rest of the treatments ($P_{A\text{-control}} = 0.06$, $P_{B\text{-A}} = 0.74$, $P_{C\text{-A}} = 0.41$ and $P_{C\text{-B}} = 0.90$).

3.3. Diversity between Conditions

The Adonis test indicated that the differences in diversity due to the treatments or plant condition were not significant for fungi ($R^2_{treatments} = 0.36$, $P_{treatments} = 0.21$, $R^2_{condition} = 0.40$, $P_{condition} = 0.32$) (Figure 2A). In bacteria, there was no evidence of the state of the plant (healthy/dead) influencing the community ($R^2_{condition} = 0.03$, $P_{condition} = 0.51$). However, the treatments did seem to exert some influence ($R^2_{treatment} = 0.17$, $P_{treatment} = 0.011$) given that treatments B (*B. amyloliquefaciens*) and C (nutrient solution) were the ones that produced the greatest changes (Figure 2B).

3.4. Rhizosphere Microbiota of Blueberry Crops

In fungi, the orders, *Pleosporales* and *Hypocreales*, were more abundant in dead plants and the order, *Capnodiales*, was more abundant in the healthy ones. The treatments produced changes at the order level, thereby increasing the presence of *Capnodiales* in all cases, especially in treatment A (*P. aeruginosa* + *B. velezensis*). This treatment also produced an increase in the orders *Eurotiales* and *Pleosporales*, and treatment B produced an increase in *Hypocreales*, which showed a decreased concentration in samples from treatment C (nutrient solution) (Figure 3A,B). The phyla, *Actinobacteria* and *Proteobacteria*, were the most abundant in healthy plants, while in dead plants we found a higher proportion of *Firmicutes* (Figure 3C). Regarding treatments, the phyla, *Myxococcota*, *Acidobacteria*, and *Proteobacteria*, were the most abundant in the control, while *Actinobacteriota* dominated the

soil from treated plants. Furthermore, the phylum, *Firmicutes*, was increased in soils from treatments B (*B. amyloliquefaciens*) and C (nutrient solution) (Figure 3D).

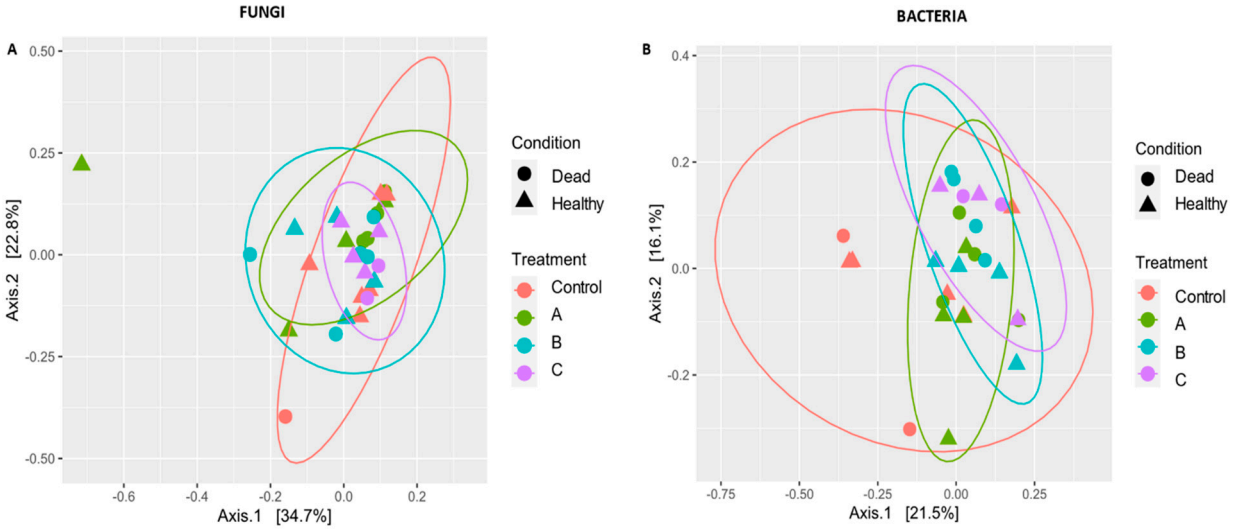


Figure 2. Beta diversity. The graphic shows the differences and the similarities between the samples due to the treatment and the condition: (**A**) PCoA (Principal Coordinates Analysis) for fungi; (**B**) PCoA for bacteria.

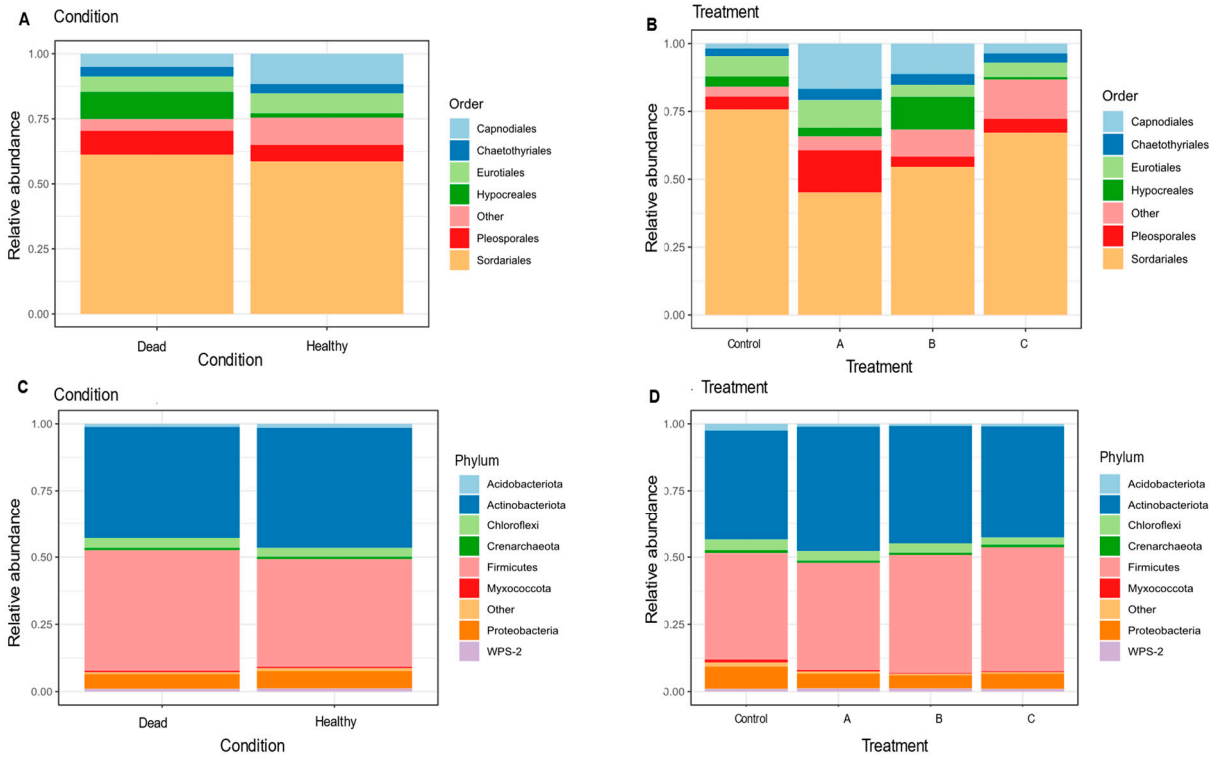


Figure 3. Relative abundances: (**A**) the relative abundance of fungi orders depending on the plant condition; (**B**) relative abundance of fungi depending on the treatment; (**C**) relative abundance of bacterial phyla depending on the plant condition; and (**D**) relative abundance of bacterial phyla depending on the treatment.

The genera, *Archaeorhizomyces* and *Fusarium*, varied in abundance depending on the plant condition: *Archaeorhizomyces* prevailed on healthy plants ($p < 0.001$, log2FoldChange = 5.1) while *Fusarium* did well on dead plants ($p = 0.01$, log2FoldChange= −2.43) (Figure 4A). For bacteria, the sole significant differences were found in the genera, *Actinospica* and *Nocardioides*,

given that the former is more abundant in healthy plants (p = 0.028, Log2FoldChange = 1.51) and the latter is more abundant in dead plants (p = 0.029, log2FoldChange = −2.95) (Figure 4B).

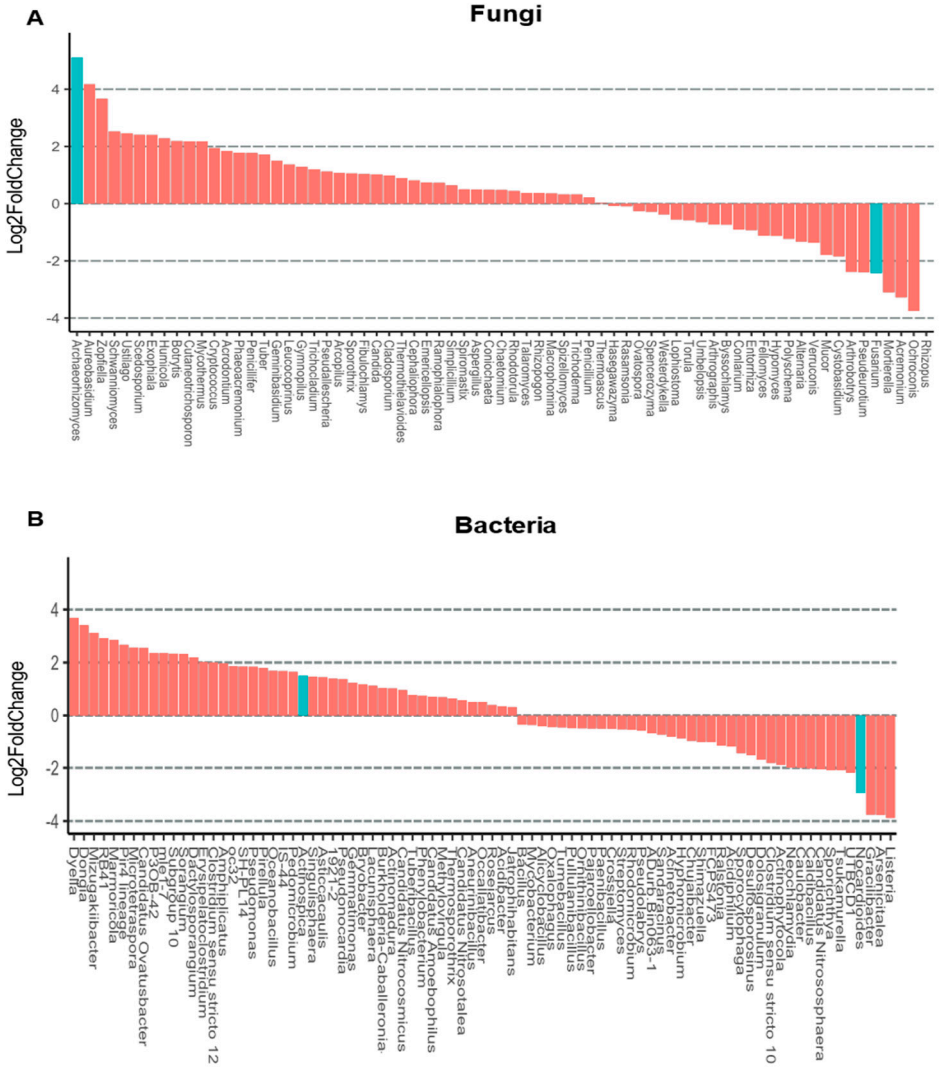


Figure 4. Changes in the relative abundance of the genera as a function of plant condition for fungi (**A**) and bacteria (**B**). The *Y*-axis represents the log2 of fold change. Red bars indicate p-value $\geq$ 0.05 and blue bars indicate p-value < 0.05. Only genera whose p-values were below 0.6 are depicted.

The soil microbiota was affected by the treatment. There were three genera of fungi and eight genera of bacteria that differed from the samples from treatment A (*P. aeruginosa* + *B. velezensis*) with respect to the control. In fungi, the genera, *Talaromyces* and *Archaeorhizomyces*, were more abundant in the treated samples, while *Rasamsonia* prevailed in the control (Figure 5A). In bacteria, eight genera changed abundances significantly, with two of them being found in greater abundances in the control than in the treated samples (*Pedomicrobium*, and *Nocardioides*) and the other six being the opposite case (*Nocardia*, *Pedosphaera*, *Roseiarcus*. *Anaeromyxobacter*, *FCPS473*, and *Burkholderia-Caballeronia-Paraburkholderia*) (Figure 6A).

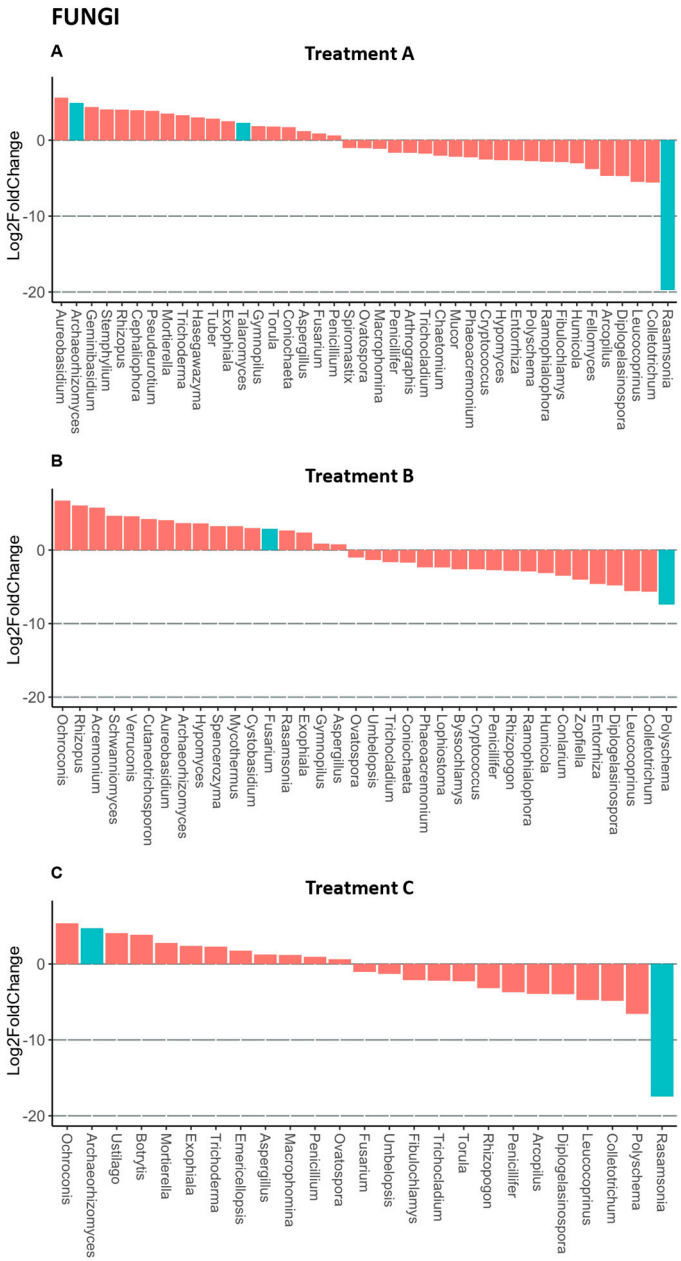


Figure 5. Changes in the relative abundance of the fungi genera as a function of treatments A (**A**), B (**B**), and C (**C**). The *Y*-axis represents the log2FoldChange. Only genera with *p*-values < 0.6 are depicted.

For treatment B (*B.amyloliquefaciens*), two genera of fungi differed with respect to the control: *Polyschema*, given that it was more abundant in the control than in the treated plants, and *Fusarium*, given that it was more abundant in the treated plants than in the control (Figure 5B). For bacteria there were 18 associated genera for all of the treatments. In the treated samples, the taxa *Conexibacter*, *Tumebacillus*, *Pullulanibacillus*, *Mycobacterium*, *FCPS473*, *Nocardia*, *Paenibacillus*, *Alicyclobacillus*, and *Acidothermus* were at higher proportions; while *Koribacter*, *Hyphomicrobium*, *Pedomicrobium*, *Sphingomonas*, *Gemmatimonas*, *Solirubrobacter*, and *Actinomadura* were more abundant in the control samples (Figure 6B).

Treatment C (nutrient solution) produced changes in 2 fungal and 21 bacterial taxa. In fungi, *Archaeorhizomyces* was more abundant in the treated plants and *Rasamsonia* was more abundant in the control ones (Figure 5C). In bacteria, the less abundant genera, with respect to the control, were *Gemmatimonas*, *Solirubrobacter*, *Nocardioides*, *MND1*, *Roseisolibacter*, *Candidatus Koribacter*, *Sphingomonas*, *Pedomicrobium*, *Hyphomicrobium*, *Candidatus Nitrososphaera*, and *Geodermatophilus*; while the most abundant taxa in the treated samples

were *Tumebacillus*, *Anaeromyxobacter*, *Ammoniphilus*, *Nocardia*, *Conexibacter*, *Burkholderia-Caballeronia-Paraburkholderia*, *Mycobacterium*, *Acidibacter*, *Acidothermus*, and *Pullulanibacillus* (Figure 6C).

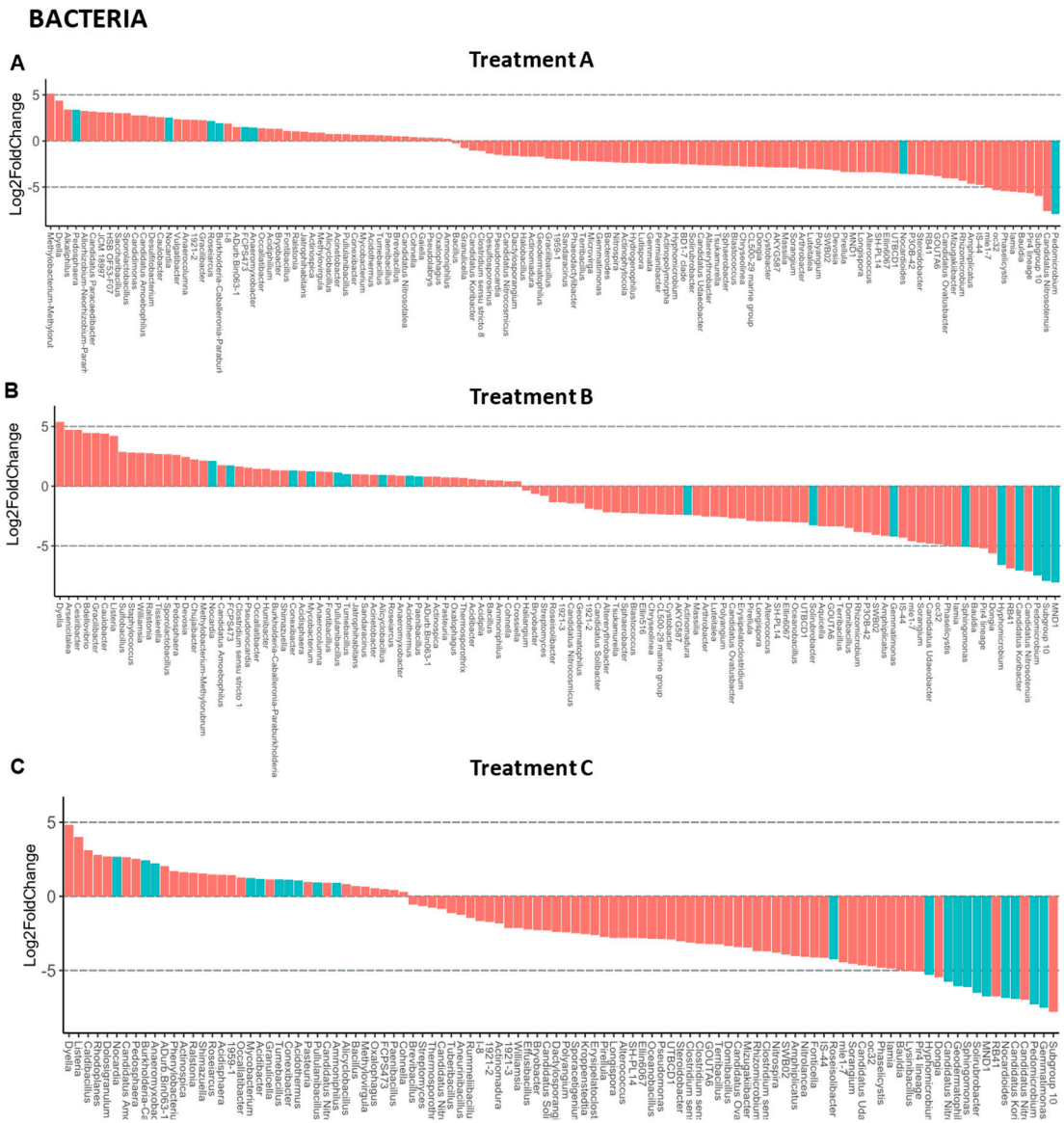


Figure 6. Changes in the relative abundance of the bacteria genera as a function of treatments A (**A**), B (**B**), and C (**C**). The *Y*-axis represents the log2FoldChange. Only genera with *p*-values < 0.6 are depicted.

4. Discussion

The pathogens that are present in soil can cause crop losses, but other microorganisms, such as PGP bacteria, can exert a suppressive effect. A better knowledge of the microbiota interactions in the soil can help to inform sustainable strategies for pathogen control. The use of metabarcoding allowed us to identify the differences in soil microbiota between healthy and dead plants that were infected with *M. phaseolina*, and to evaluate the changes in the soil microbiota that were associated with the different control strategies assayed.

High soil microbial diversity has been associated with increased crop yield and resistance to pathogen attacks [58,59]. The higher diversity observed in live plants with respect to dead plants supports this hypothesis. Fungal diversity was low, and it was not influenced by either plant condition or treatment (Figure 1), meanwhile the bacterial microbiota was responsive to treatments (Figure 2).

Healthy and dead plants differed in their microbiota composition. The fungal order, *Capnodiales*, was more abundant around healthy plants; it includes diverse fungi with different lifestyles and nutrition pathways [60] (Figure 3A). The bacterial phyla, *Proteobacteria* and *Actinobacteria*, were predominant in healthy plants (Figure 3C). Many groups within these phyla have been associated with PGP activities and are related to plant development [61]. In dead plants, the fungal orders, *Pleosporales*, which contains parasites, epiphytes, or endophytes, and *Hypocreales*, which harbours genera that have been employed for biocontrol [62,63] (Figure 3A), were the most abundant. The bacterial phylum, *Firmicutes*, was slightly more abundant in dead plants (Figure 3C). That fact could contrast with the PGP activities associated with some of the strains that belong to this phylum, which are well known and have been used as biocontrol microorganisms [64].

At the genus level, *Fusarium*, a blueberry pathogen [65], was the most abundant in dead plants, and *Archaeorhizomyces*, a non-pathogen ubiquitous fungus commonly associated with plant roots [66], was most common in healthy plants (Figure 4A). The high presence of *Fusarium* in dead plants is cohesive with the positive interaction that this pathogen establishes with *M. phaseolina;* the latter could enhance the severity of the former [16]. We did not report a larger abundance of *M. phaseolina* in dead plants, contrary to our expectations. Perhaps, it could be explained by the fact that this pathogen is an endophyte and the studied samples were collected from soil [10]. For bacteria, the genus *Nocardioides*, which is most abundant in dead plants, is related to mineral fixation and organic material degradation [67]. It has also been associated with the resistance of *Fusarium oxysporum* and *M. phaseolina* since it contains strains with PGP activities and antifungal producers [68,69]. The larger presence of this genus in dead plants seems contradictory to what is known about this genus, although there may be undescribed functions that could explain this fact. On the contrary, the genus *Actinospica*, which has been used as a biocontrol [70], was the most abundant in healthy plants (Figure 4B).

The treatments produced changes in the soil microbial composition, especially treatments B (*B. amyloliquefaciens*) and C (nutrient solution) (Figure 2B). Biocontrol approaches have been described as effective strategies for the prevention of pathogen infections in many crops, and other studies support the use of the microorganisms employed in treatments A and B. *Pseudomonas aeruginosa*, the microorganism employed in treatment A, has been shown to have PGP activities and a strong antagonism against worldwide pathogens, such as *M. phaseolina* and *F. oxysporum* [71]. Furthermore, the genus *Bacillus*, which was used in treatments A and B, has been employed as a biocontrol against *M. phaseolina*, *Fusarium* spp., and *Rhizoctonia solani*, and has had good results in other crops [72].

At the fungal level, all treatments led to an increase in *Capnodiales*, which predominated in healthy plants. Soils under treatment A (*P. aeruginosa* + *B. velezensis*) were also abundant in the orders, *Eurotiales* and *Pleosporales*, given that the latter was very abundant in dead plants. *Eurotiales* harbors species from *Penicillium* or *Aspergillus*, which contain strains used in biocontrol [73–75]. Treatment B (*B. amyloliquefaciens*) increased the abundance of *Hypocreales*, which was also very abundant in dead plants (Figure 3B). At the genus level, treatment A (*P. aeruginosa* + *B. velezensis*) produced an increase in *Talaromyces*, which contains strains with PGP and biocontrol activities [76–78], and in *Archaeorhizomyces*, which was the most abundant in healthy plants (Figure 5A). *Archaeorhizomyces* was also increased in soils under treatment C (nutrient solution) (Figure 5C). Treatment B (*B. amyloliquefaciens*) led to a remarkable increase in *Fusarium*, whose presence was also higher in dead plants (Figure 5B).

The bacterial soil composition was also modified after the treatments employed. In all treated soils, *Actinobacteriota* were observed in larger abundances (Figure 3D). The abundance of *Actinobacteriota* was also greater in healthy plants, so some of their strains could have favorable properties for plant development or resistance. In treatment A (*P. aeruginosa* + *B. velezensis*), *Pedomicrobium*—which contains strains related to nitrogen fixation [79]—and *Nocardioides* reduced their presence with respect to the control soils. Six genera were increased in soils under treatment A, two of them (*Roseiarcus* and *Anaeromyxobacter*) with

strains related to nitrogen fixation [79,80], two *FCPS473* and *Pedosphaera*) with strains able to degrade xenobiotics or organic material [81,82], and two with strains that could act as pathogen antagonists: *Nocardia*, which can inhibit *Fusarium* and also has PGP activities [83], and *Burkholderia-Caballeronia-Paraburkholderia*, which can act against other pathogens [84] (Figure 6A). Treatment C significantly reduced the abundance of five genera with strains implicated in N-fixation (*MND1*, *Roseisolibacter*, *Pedomicrobium*, *Hyphomicrobium*, *Candidatus Nitrososphaera*, and *Geodermatophilus*) [79,85–87], three with strains that could act as PGP (*Gemmatimonas*, *Solirubrobacter*, and *Sphingomonas*) [88–90], and one that could act as a pathogen antagonist (*Nocardioides*). On the contrary, in treated soils there were two genera with strains implicated in nitrogen fixation (*Anaeromyxobacter* and *Acidothermus*) [79], two strains that could act as PGP (*Conexibacter* and *Acidibacter*) [91,92], two that contain potential pathogen antagonists (*Nocardia* and *Burkholderia-Caballeronia-Paraburkholderia*), one with strains that could degrade xenobiotics (*Mycobacterium*) [93], a genus that contains extremophiles (*Pullulanibacillus*) [94], and another with strains that can use oxalacetate (*Ammoniphilus*) [95] (Figure 6C).

The presence of bacteria that degrade xenobiotics in any of the treatments compared to the control and the disappearance of bacteria with functions involved in nitrogen fixation in the case of treatment C (nutrient solution) is striking. This treatment contains assimilable nitrogen in its composition, which may be related to the displacement of nitrogen fixers in the treated samples. As indicated by the β-diversity, treatments B (*B. amyloliquefaciens*) and C (nutrient solution) were the ones that modified bacterial composition the most. Given the changes and the characteristics attributed to the genera, it seems that treatment A (*P. aeruginosa* + *B. velezensis*) is the most favorable at both predicted fungal and bacterial activities. The results presented here indicate the short-term effect of the treatments. To know the true effect of the tested treatments, we should extend the trial over time, as it has only been applied during one season, and the blueberry is a perennial crop. Furthermore, this study has shown the positive relation between *M. phaseolina* and *Fusarium*. The biochemical fundamentals of this relation should be studied for developing a better understanding and for establishing good methods for the control of both infections.

5. Conclusions

A higher diversity was observed in healthy plants when compared with dead ones. Dead plants, all of which were infected with *M. phaseolina*, had a larger abundance of *Fusarium* spp., another blueberry pathogen, suggesting a synergy of both pathogens in plant infection. Some taxa changed in abundance according to the health state of the plant, although the treatments had the strongest effect on the microbiota, especially biocontrol treatment A. Our work has revealed the fungal and bacterial diversity patterns associated with blueberry crop soils. Future steps could be directed at elucidating the biocontrol mechanisms of some of the proposed bacteria or from newly screened bacteria, and could also contribute to understanding the complex microbial interactions that occur in the rhizosphere and in the interior of the plant tissues by using metagenomics or metatranscriptomics.

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