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Article

Non-Target Effects of *Trichoderma*- and *Bacillus*-Based Products on the Citrus Microbiome

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Abstract

Microbial communities associated with the rhizosphere and phyllosphere are recognized as fundamental components influencing essential plant processes, including nutrient acquisition, growth promotion, and tolerance to stress. Biological control agents (BCAs), such as *Trichoderma* spp. and *Bacillus* spp., are widely applied in citrus crops. However, while BCAs effectiveness against plant pathogens is widely established, their resulting impact on indigenous, non-target bacterial and fungal communities remains poorly understood. The aim of this study was to evaluate the non-target effects of two commercial microbial formulations—one containing *Trichoderma asperellum* ICC 012 and *T. gamsii* ICC 080, and the other *Bacillus amyloliquefaciens* QST 713—on the resident microbiomes of *Citrus volkameriana* seedlings by using the amplicon-based metagenomic analysis, targeting the 16S rRNA and ITS1 regions. The application of the *Trichoderma* formulation as a soil drench in the rhizosphere resulted in minimal changes to the overall composition and diversity (α- and β-diversity) of the bacterial communities. This stability is considered a desirable trait for overall soil health. However, specific taxonomic changes were observed, such as a notable decrease in the genus *Rhodococcus* (0.4% vs. 1.5% in controls) among bacteria. In the fungal communities, the treatment led to a significant shift in phylum relative abundance, characterized by an increase in Basidiomycota (38% vs. 28% in controls) and a corresponding decrease in Ascomycota (51% vs. 56% in controls). Successful colonization was confirmed by a substantially higher relative abundance of the inoculated *Trichoderma* genus compared to control plants (1.4% vs. 0.1% in controls). Conversely, the foliar application of the *Bacillus* product induced a substantial restructuring of the phyllosphere bacterial community. This treatment caused a statistically significant reduction in bacterial α-diversity and a clear differentiation in community composition (β-diversity) relative to untreated controls. The successful colonization by the BCA resulted in the dominance of the *Bacillus* genus in the treated samples (27% vs. 2% in controls). Importantly, this ecological shift was accompanied by the enrichment of other beneficial bacterial taxa, including *Sphingomonas* (15% vs. 4% in controls) and the *Burkholderia-Caballeronia-Paraburkholderia* group (4% vs. 2% in controls). While fungal phyla abundances remained generally stable in the phyllosphere, specific genera such as *Cladosporium* (15% vs. 23% in controls) and *Symmetrospora* (21% vs. 13% in controls) prevailed post-treatment. In conclusion, these findings highlight the importance

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of considering non-target microbiome shift when implementing microbial biocontrol strategies in citrus production systems, since in this study was demonstrated that commercial BCAs exert a markedly differential influence based on the compartment of application: *Trichoderma* promoted ecological stability in the rhizosphere, whereas *Bacillus* induced a directional community shift in the phyllosphere.

Keywords: *Citrus volkameriana*; biological control agents; phyllosphere microbiome; rhizosphere microbiome; amplicon-based metagenomics

1. Introduction

Plants function as holobionts, complex ecosystems comprising the host plant and its associated microbial communities, collectively known as the microbiome [1,2]. The term microbiome has recently been revisited and refined based on the original definition as the community of microbes (microbiota) combined with their theater of activities, which encompasses macromolecules, mobile genetic elements, as well as microbial metabolites [3]. These microbial communities colonizing key plant compartments, namely the rhizosphere (root–soil interface), phyllosphere (aerial plant surfaces), and endosphere (internal tissues), are fundamental to supporting plant growth, health, and resilience [4]. They are implicated in essential processes like growth promotion, nutrient acquisition, stress tolerance, induction of systemic resistance, and direct protection against pathogens [5]. Numerous studies have examined how plant characteristics and environmental factors shape plant-associated microbial communities [6–8] determining which populations thrive and how they interact with the host, with some conditions favoring beneficial groups and others supporting pathogens.

This holistic framework is crucial for understanding the biology of key crops like citrus (*Citrus* L., Rutaceae), which rank among the most important fruit crops globally due to their economic value and broad consumer demand [9]. They are used fresh, processed into juices and concentrates, incorporated into various food products and beverages, and serve as key sources of essential oils [9]. Within the EU, Italy is the second-largest citrus producer after Spain, and it is the world leader in organic citrus cultivation, with nearly 40,000 hectares dedicated to organic orchards, representing about 45% of the global organic citrus area [10]. In Italy, as in other major EU citrus-producing countries, oranges are the most cultivated citrus crop, followed by mandarins and lemons [11].

An increasing number of studies have begun to characterize the citrus microbiome as a key determinant of plant health [2,12,13]. This is supported by the fact that the microbial communities in the rhizosphere, endosphere, and phyllosphere of citrus plants are distinct and influenced by various factors, including plant diseases [14–17].

Different fungal diseases significantly constrain citrus production in the European citrus-producing countries, including Alternaria brown spot (ABS) caused by *Alternaria* spp., anthracnose caused by *Colletotrichum* spp., canker-disease caused by different fungal pathogens [18–20] and the vascular disease named Mal secco caused by *Plenodomus tracheiphilus* (Petri) Gruyter, Aveskamp & Verkley (syn. *Phoma tracheiphila*) [21,22]. Among these, Mal secco disease represents one of the most serious threats to the citrus production, particularly affecting lemon. The use of fungicides, such as copper-based compounds, has been progressively restricted in response to European Union policies aimed at reducing the environmental footprint, particularly concerning effects on soil biodiversity [23–26]. With copper being the only chemical option permitted in organic agriculture, this reduction poses a major challenge for disease management.

In this context, the increasing need to limit chemical pesticides use has driven the exploration of more sustainable alternatives, including biological control agents (BCAs), beneficial microorganisms capable of suppressing pathogens through a variety of mechanisms [27–30]. BCAs offer a highly promising alternative for crop protection, although their success in the field can be limited by local biotic and abiotic factors [31,32]. *Bacillus* spp. and *Trichoderma* spp. are among the most widely authorized BCAs in the EU for the control of bacterial and fungal diseases [33]. The efficacy of various *Trichoderma* and *Bacillus* strains in controlling different citrus pathogens has been widely demonstrated in previous studies [34–46].

Among *Bacillus* species, *Bacillus amyloliquefaciens* and *B. subtilis* are the most tested bacterial BCAs against citrus pathogens in Italy [37,38,42–44]. They produce a wide range of beneficial molecules that can induce or elicit plant defenses, as well as directly compete with or antagonize pathogens. Moreover, they are reported as frequent endophytic bacteria of citrus, and their ability to survive and colonize internal tissues make them suitable in the control of vascular plant disease [27]. *Trichoderma*, also known by its sexual morph *Hypocrea* (Hypocreaceae, Hypocreales), is a soil-borne fungal microorganism widely used as biocontrol agent for its ability to produce a wide array of beneficial molecules that can directly compete with, antagonize and mycoparasitize fungal pathogens, as well as indirectly induce plant defense responses, promote plant growth and enhance soil productivity [28,47,48]. Different plant protection products containing *Bacillus* and *Trichoderma* species have been registered worldwide [27,28]. Among these, *B. amyloliquefaciens* strain QST 713 (formerly *B. subtilis*, Serenade® Aso), and *T. asperellum* ICC 012 + *T. gamsii* ICC 080 (Remedier®), already registered in Italy [49], were recently evaluated for control of Mal secco disease (MSD), a vascular tracheomycosis caused by the fungus *Plenodomus tracheiphilus* (previously *Phoma tracheiphila*) which severely threatens lemon cultivation in the Mediterranean basin [42–44,50] and that has been shown to alter soil and citrus microbial communities [17,51]. While the efficacy of BCAs, including *Trichoderma* and *Bacillus*, against citrus pathogens is well documented [42–44], their non-target effects on the resident microbial communities in the rhizosphere and phyllosphere remain largely unexplored [51]. Assessing the impact of biocontrol agents on indigenous microbial assemblages is essential to fully understand their mode of action and ecological behavior [52–55]. A deeper understanding of the complex interactions among microorganisms, and between microorganisms and the host, is fundamental for the development of more effective and sustainable biocontrol strategies since the plant-associated microbiome plays a crucial role in plant health, disease suppression and stress tolerance [52,56]. To address this gap, the present study aims to investigate the broad-spectrum effects of two commercial biocontrol formulations on the resident bacterial and fungal communities in specific plant compartments, in the absence of the target pathogen, through the amplicon-based metagenomics. Specifically, we characterized the microbial communities in the rhizosphere of *Citrus volkameriana* seedlings treated with *T. asperellum* ICC 012 and *T. gamsii* ICC 080 applied as a soil drench, and in the phyllosphere of plants treated with *B. amyloliquefaciens* strain QST 713 applied as a foliar spray, both applied following their authorized usage protocols.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

To evaluate the non-target effect of two commercial *Trichoderma*- and *Bacillus* based-products, two independent experiments were performed from October 2023 to March 2024 on one-year-old seedlings of the rootstock *Citrus volkameriana* in a commercial nursery located in Mascali (Catania province, Eastern Sicily, Italy) under the nursery's standard commercial growing conditions, except that no microbial treatments were applied. The site is located at 37.749015° N latitude and 15.198351° E longitudes at an altitude of 18 m above sea level, an area belonging to a Mediterranean climate characterized by mild winters and hot summers (Csa Köppen climate classification) with minimum and maximum temperatures varying from 5.7 to 20.6 °C and from 13.8 to 30.6 °C, respectively, based on climatic data measured by a climate station of Riposto (Catania, Italy) belonging to the regional Agro-meteorological Information Service (SIAS) [57]. Plants were maintained in plastic pot of 17 cm diameter with a volume of 3 L, filled with a commercial substrate (40% sand + 30% blond peat + 15% coconut fiber + 5% expanded clay) previously fertilized with the organic fertilizer Ficote® Total 8-9M (ICL Italia, Treviso, Italy) (containing 17% N, 9% P_2O_5, 11% K_2O) at 3 kg m^{-3} and with the micronutrient fertilizer Micromax® Premium (ICL Italia, Treviso, Italy) (containing 14% MgO, 42.2% SO_3, 15% Fe, 1% Cu, 1% Zn, 2.5% Mn, 0.02% B, and 0.04% Mo) at 0.3 kg m^{-3}. During the experiment, additional fertilization was applied once per month using NPK Original Gold® (Compo Expert Italia, Cesano Maderno, Italy) (containing 15% N, 9% P_2O_5, 15% K_2O, 22.5% SO_3, 2% MgO, 0,3% Fe, 0.01% B, 0.002% Cu, 0.01% Mn, 0.002% Zn) at 0.0045 kg per plant. In total, eight applications of each BCA were performed before sample collection in early March. Applications were generally carried out once per month, except in January, when two treatments were applied. Ten-plant plots were arranged in a randomized complete block design, with four blocks, each including 40 plants, with a total of 160 plants per treatment. The block size was 250 (length) × 115 cm (width). Guard trees were located between plots within rows, and one guard row was located between treated rows.

2.2. *Trichoderma*-Based Product Application, Plant Sample Collection and Processing

A *Trichoderma*-based product, containing *T. asperellum* ICC 012 + *T. gamsii* ICC 080 [active ingredient (a.i.) $3 \times 1.\ 0^{7}$ CFU g^{-1}, Remedier®, Gowan Italia], was applied as root drench according to the manufacturer's instruction for citrus crop with the aim to assess its non-target effect on the rhizosphere microbial communities. Thus, Remedier® was prepared 24 h prior to application in order to activate conidial germination of *Trichoderma* spp., then diluted in tap water at a concentration of 167 g hL^{-1} and applied to the soil of each plant at volume of 125 mL (corresponding to 5 L per block) using a shoulder sprayer. Control plants consisted of plants treated with the same amount of water. *C. volkameriana* seedlings were sampled in March 2024 two weeks after the last soil drench of the *Trichoderma*-based product. More in detail, composite rhizosphere samples (roots with their adhering soil) were collected from three plants per experimental block. Three such composite samples were processed for each randomized block, resulting in a total of 12 replicates. Samples were processed according to Anzalone et al. [58,59] with minor modifications. Briefly, loose, non-adhering soil was removed from the roots by gentle shaking. Subsequently, five grams of roots with adhering soil were transferred to a sterile 50 mL centrifuge tube containing 20 mL of saline solution (0.85% NaCl) and vortexed thoroughly for 2 min.

2.3. *Bacillus*-Based Product Application, Plant Sample Collection and Processing

A *Bacillus*-based product, containing *B. amyloliquefaciens* QST 713 (formerly *B. subtilis*) (a.i 1×10^{12} CFU kg^{-1}, Serenade® Aso, Bayer CropScience) was applied as foliar spray according to the manufacturer's instruction for citrus crop with the aim to assess its non-target effect on the phyllosphere microbial communities. Thus, Serenade® Aso was diluted in tap water at a concentration of 533 mL hL^{-1}, and 25 mL (corresponding to slightly more than 1 L per block) were distributed over the leaf using a shoulder sprayer. Untreated control plots were arranged according to both distribution methods using only tap-water. The same amount of water was applied in each control plant. Sampling was conducted in

March 2024 two weeks after the last foliar spray application of the *Bacillus*-based product. To obtain the phyllosphere samples, the protocol of Li et al. [60] was adapted with some modifications. For each replicate, six leaves from two plants were pooled into a 1 L Erlenmeyer flask containing 200 mL of saline solution (0.85% NaCl). The samples were incubated for 90 min at 200 rpm on an orbital shaker and then sonicated for 7 min in a Branson 52 sonicator. The resulting suspension from each replicate was concentrated by vacuum filtration through sterile gridded cellulose nitrate membrane filter (Axiva Sichem, Delhi, India) with 0.22 μm pore size using a multi-place stainless steel filtration manifold (Sartorius, Göttingen, Germany). The filter was carefully aseptically removed from the manifold using forceps, and the retained microbial biomass was eluted and resuspended in 5 mL of sterile saline buffer (0.85% NaCl) for subsequent centrifugation.

2.4. DNA Extraction

The suspensions from both the rhizosphere and phyllosphere samples were aliquoted into 2 mL microcentrifuge tubes and centrifuged at 13,000 rpm and 4 °C for 30 min. The supernatant was discarded, and the pellets were stored at −80 °C. Genomic DNA was subsequently extracted from the pellets using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration and purity of the extracted DNA were evaluated using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

2.5. Library Preparation and Amplicon Sequencing

Library preparation and amplicon sequencing were performed by IGA Technology Services (Udine, Italy). The V3–V4 hypervariable region of the bacterial 16S rRNA gene was amplified using the 16S-341F and 16S-805R primers for bacterial community profiling [58]. The ITS1 region of the fungal rRNA operon was amplified with ITS1/ITS2 primers for fungal community profiling [61]. Peptide nucleic acid (PNA) clamping was applied during the initial amplification step to specifically block amplification of host chloroplast and mitochondrial 16S rRNA gene sequences. Sequencing of the 16S and ITS libraries was performed on an AVITI system (Element Biosciences, San Diego, CA, USA) using a 300 bp paired-end configuration. The paired-end reads were then merged to create high-quality, full-length sequences, ensuring reliable and precise taxonomic identification. The ASVs obtained were compared with those in GenBank database using BLAST (v.2.17.0) [62,63]. BLAST search was performed by restricting the search to sequences belonging to the *Bacillus* and *Trichoderma* species used in this study.

2.6. Bioinformatics and Statistical Analysis

Taxonomic assignment of ASVs was performed through the 16S SILVA 138 database [64] for the 16S reads, and the UNITE database (version 9.0, all eukaryotic dynamic) [65] for ITS reads. Unassigned ASVs and those identified as chloroplasts and mitochondria were removed from the 16S ASV table. Regarding the ITS ASV table, only taxa classified as belonging to the Fungi kingdom were retained. The α- and β-diversity analyses were performed using the phyloseq package (version 3.17) [66] in R (v. 4.0.2) [67].

The α-diversity within each sample was determined using Observed richness and the Shannon diversity indexes. Differences in α-diversity between treated and untreated samples were assessed using the Kruskal-Wallis test followed by pairwise comparisons. β-diversity was characterized using Bray-Curtis dissimilarity and visualized through Principal Coordinate Analysis (PCoA) to evaluate compositional differences both within and among sample groups. The Permutational multivariate analysis of variance (PERMANOVA) test, conducted using the vegan package [68] in R, was considered to assess statistical differences between sample groups with 999 permutations. Differential abundance analysis was performed with DESeq2 (v. 1.40.2) [69] to identify bacterial and fungal ASVs that were significantly enriched or depleted in treated samples relative to non-treated controls. Statistical significance was evaluated using adjusted *p*-values calculated with the FDR correction method.

Co-occurrence networks were generated using the ASVs for the rhizosphere and phyllosphere compartments. For each compartment, correlations were evaluated for both control and treatment conditions. Networks were generated with Spearman's rank correlation calculated through the psych R package [70]. Only the ASVs with a relative abundance greater than 0.04% and occurring in at least 50% of the specimens in the bacterial and fungal data sets were considered. Gephi (v 0.10) [71] was adopted to visualize and analyze the networks. Both positive and negative correlations greater than or less than 0.60 and -0.60, respectively, were plotted in networks with a p value < 0.001. Fruchterman Reingold algorithm was used for the network layout.

3. Results

3.1. General Characteristics of Sequencing Datasets

Bacterial and fungal communities were obtained from the rhizosphere and phyllosphere of *C. volkameriana* seedlings treated either by soil drench with *T. asperellum* ICC 012 + *T. gamsii* ICC 080 or by leaf application of *B. amyloliquefaciens* QST 713, respectively. The treatments were compared to rhizosphere and phyllosphere samples obtained from untreated plants. AVITI sequencing of the bacterial 16S rRNA gene and the fungal ITS region from treated and untreated rhizosphere produced 8,478,728 and 13,867,556 raw reads, respectively. From the treated and untreated phyllosphere samples, AVITI sequencing of the bacterial 16S rRNA gene and the fungal ITS region produced 3,599,906 and 2,516,159 raw reads, respectively. After quality filtering (length trimming, denoising, exclusion of chimeric sequences, singletons, chloroplasts, and nonmicrobial reads), a total of 3,297,932 and 5,494,760 reads were obtained for the bacterial and fungal communities from rhizosphere samples, respectively, while a total of 1,323,465 and 2,516,159 reads for the bacterial and fungal communities, respectively, were obtained from phyllosphere samples. The information related to the samples assessed through amplicon-based metagenomics and their respective raw sequences are available on Zenodo (see Data Availability Statement).

3.2. Modulation and Stability of Rhizosphere Microbial Communities Following Trichoderma Treatment

3.2.1. Effect of *Trichoderma* Treatment on Rhizosphere Microbial Communities

The impact of the soil drench applications with the *Trichoderma*-based formulation (containing *T. asperellum* ICC 012 + *T. gamsii* ICC 080) on the microbial communities of *C. volkameriana* rhizosphere was assessed. Analysis of α-diversity, which captures complexity within individual samples, revealed no significant effects of the treatment. Specifically, the richness (as measured by the Observed ASV index) and the Shannon diversity index of both bacterial and fungal communities in the rhizosphere showed no statistically significant differences when compared to the untreated control group (Kruskal-Wallis test, $p > 0.05$, FDR adjustment) (Figure 1A,B).

Furthermore, to evaluate the differences in overall community structure between the sample groups, we conducted a β-diversity analysis, based on the Bray-Curtis dissimilarity index and depicted through a Principal Coordinate Analysis (PCoA) plot. The resulting plots depicted a strong similarity in the composition of both bacterial and fungal communities regardless of the treatment (Figure 1C,D). Permutational multivariate analysis of variance (PERMANOVA) confirmed that this clustering pattern was not statistically significant, confirming that the *Trichoderma* treatment did not induce a significant shift in the overall composition of the rhizosphere microbial communities R^2 "Treatment" = 0.04 in bacterial communities, R^2 "Treatment" = 0.06 in fungal communities, $p > 0.05$, FDR-adjusted).

Overall, the soil drench application of *T. asperellum* ICC 012 and *T. gamsii* ICC 080 induced only minimal changes to the overall composition of the bacterial communities in the *C. volkameriana* rhizosphere. Across all samples, a total of 41 bacterial phyla were identified, with the community dominated by Proteobacteria, Planctomycetota, Actinobacteriota, Verrucomicrobiota, and Acidobacteriota; these five phyla collectively constituted more than 70% of the relative abundance in both treated and control plants. Despite the overall stability, subtle shifts in the relative abundance of the most abundant phyla were detected. In treated plants, we observed a slight decrease in Proteobacteria (31.7% vs. 32.5%), Actinobacteriota (10.0% vs. 11.6%), and Bacteroidota (4.5% vs. 5.1%), while Planctomycetota (11.6% vs. 10.5%) and Acidobacteriota (10.6% vs. 9.0%) were marginally increased compared to the control group (Figure 1E). The most substantial change was observed at a finer taxonomic resolution: the relative abundance of the genus *Rhodococcus* was reduced approximately fourfold in treated plants (0.4%) compared to controls (1.5%) (Figure 1F).

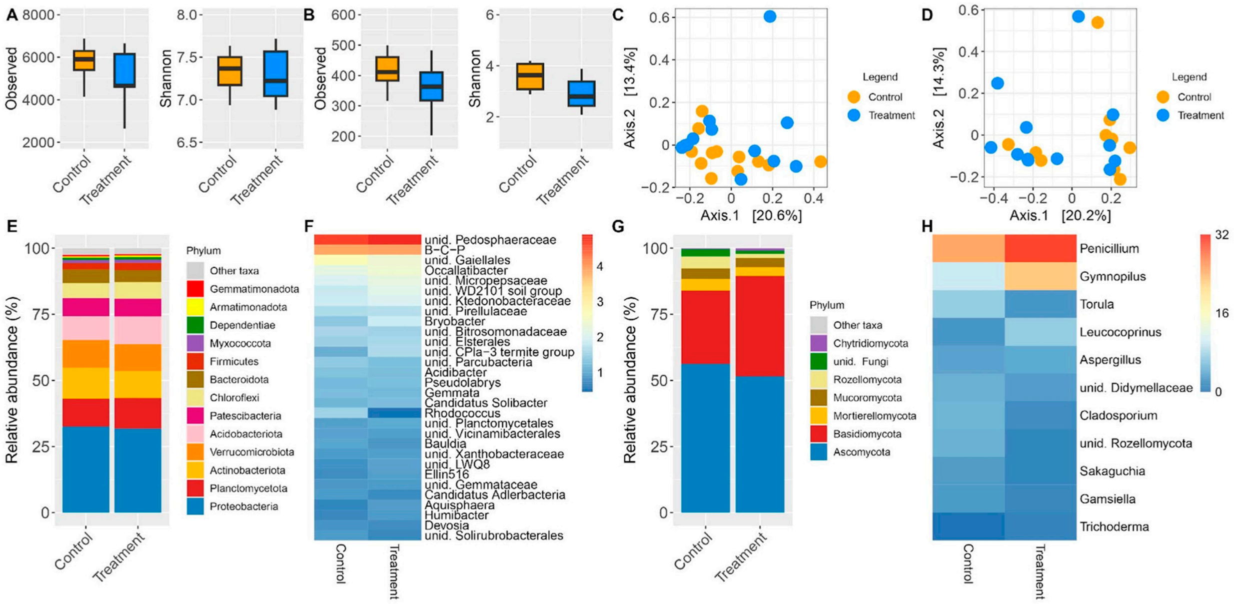

Figure 1. Rhizosphere microbial communities of *Citrus volkameriana* plants treated by soil drenching with *Trichoderma asperellum* ICC 012 and *Trichoderma gamsii* ICC 080 (Remedier®) (Treatment) and untreated plants (Control). α-diversity analysis (Observed and Shannon indices) of the bacterial (**A**) and fungal (**B**) communities. PCoA plot of β-diversity analysis based on the Bray-Curtis dissimilarity index of bacterial (**C**) and fungal (**D**) communities. Relative abundance of the most abundant bacterial phyla (**E**) and genera (**F**) (> 1.5% on the overall) and the most abundant fungal phyla (**G**) and genera (**H**) (> 1% on the overall).

The impact of *T. asperellum* ICC 012 and *T. gamsii* ICC 080 treatment on the fungal community was also assessed. A deeper insight into the rhizosphere fungal communities revealed the presence of 20 fungal phyla, dominated by Ascomycota and Basidiomycota in both control and treated plants. However, the treatment affected their relative abundances, as a notable decrease in the relative abundance of Ascomycota was observed in treated plants (51%) compared to the control group (56%). In contrast, the relative abundance of Basidiomycota increased substantially in the treated rhizosphere (38% vs. 28% in controls) (Figure 1G). Minor phyla, including Mortierellomycota (3.3% vs. 4.5%)

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and Mucoromycota (3.5% vs. 3.9%), were slightly reduced following the treatment (Figure 1G). At the genus level, *Penicillium* (Ascomycota) remained the most abundant genus in both conditions, with a higher relative abundance in treated plants (30% vs. 25%) (Figure 1H). Other prevalent genera included *Gymnopilus*, *Torula*, *Leucocoprinus*, *Aspergillus*, and unidentified Didymellaceae. *Trichoderma* sp. was found higher in treated rhizosphere soil (1.4%) than in control plants only treated with water (0.1%) (Figure 1D).

3.2.2. Abundance of *Trichoderma* and Differential Abundance Analysis of the Microbial Communities in the Rhizosphere

Despite the absence of significant changes in overall bacterial and fungal α- and β-diversity, the application of *Trichoderma*-based products resulted in a slight but consistent increase in the relative abundance of the genus *Trichoderma* in treated plants (1.4%) compared to controls (0.1%). This pattern was further supported by ASV84 read abundance, with *Trichoderma* showing consistently higher representation in treated samples, where read counts reached several thousand in individual samples. In contrast, control samples exhibited markedly lower abundances, with read counts generally remaining below one hundred on average (Figure 2A).

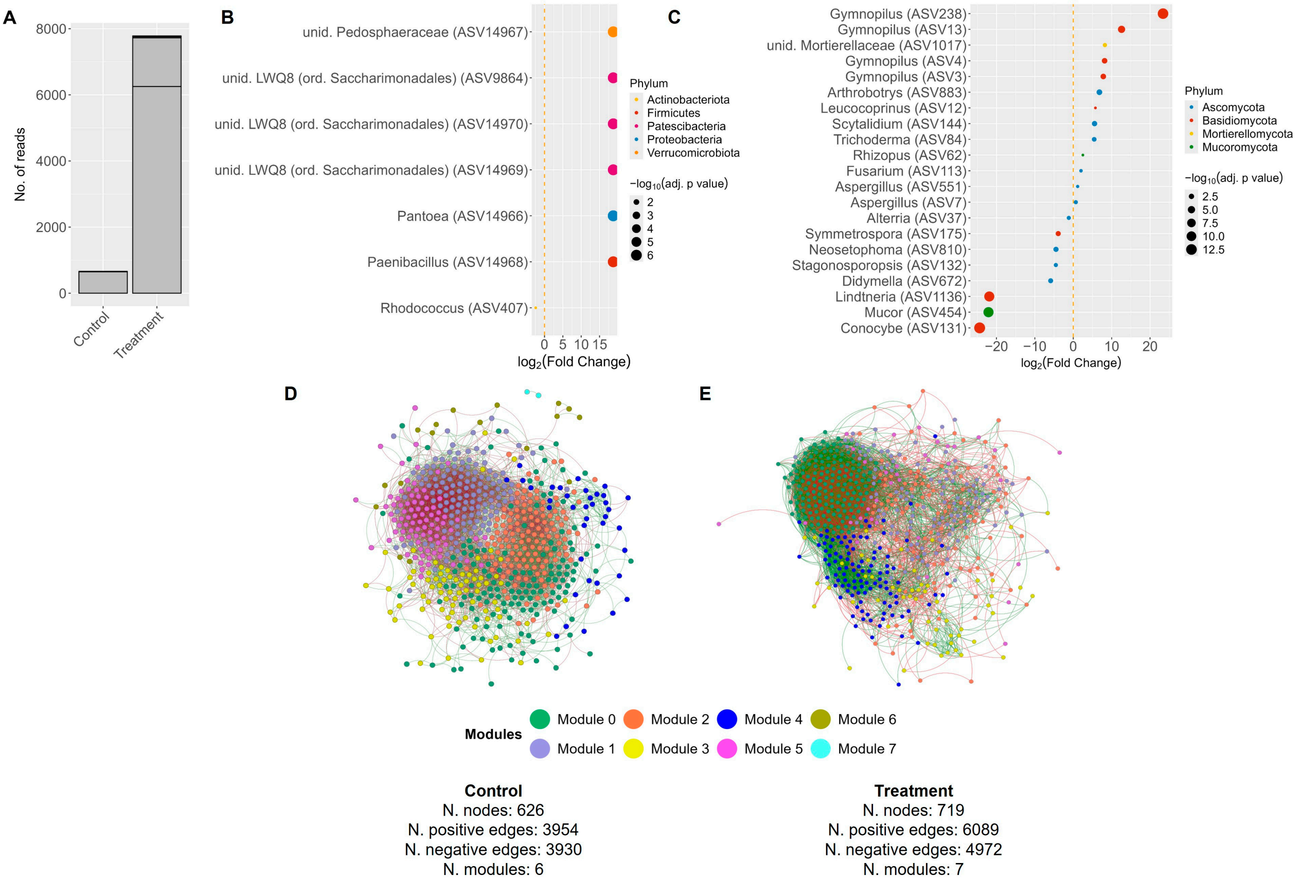

Figure 2. Cumulative absolute abundance of ASV84 identified as *Trichoderma*, significantly enriched in rhizosphere treated with *Trichoderma asperellum* ICC 012 + *Trichoderma gamsii* ICC 080 (Remedier®) (Treatment) (**A**). Differentially abundant bacterial (**B**) and fungal (**C**) ASVs of the rhizosphere, statistically significant (–log10 (p) > 1.30, FDR) enriched or depleted, in the treated plants against the untreated ones. Co-occurrence networks of bacterial and fungal ASVs in the control (**D**) and treatment (**E**) samples. Green and red edges represent positive and negative statistically significant interactions ($p < 0.001$), respectively.

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Accordingly, differential abundance analysis revealed only a limited number of significant changes, with seven bacterial and 24 fungal ASVs differing between treated and control rhizospheres. Notably, six differentially abundant bacterial ASVs were enriched in the treatment. These included four ASVs not taxonomically identified at the genus level, assigned to the phyla Patescibacteria and Actinobacteriota, as well as two ASVs identified as *Pantoea* (Proteobacteria) and *Paenibacillus* (Firmicutes) (Figure 2B). In contrast, a single ASV classified as *Rhodococcus* was significantly depleted in the treatment group relative to the control, consistent with the relative abundance analysis, which showed higher representation of this genus in control samples (Figure 2B, Supplementary Table S1).

Regarding the fungal communities, the *Trichoderma* ASV (ASV84) was found enriched in the treatments (Figure 2C, Table S2). This ASV was the most abundant among the other ASVs identified as *Trichoderma* and in the treated rhizosphere showed 100% identity with different *T. asperellum* strains, and more specifically 98.65% with *T. asperellum* strain ICC 012 (Acc. N. MU868072.1). Other two ASVs in the treated rhizosphere were ASV571, showed 100% identity with different *T. asperellum* strains and 99.55% with *T. asperellum* strain ICC 012 (Acc. N. MU868072.1), whereas ASV4552 showed 100% identity with *T. gamsii* strains, and more specifically, also with *T. gamsii* strain ICC 080 (Acc. N. GQ351597.1). However, *T. asperellum* ASVs (ASV65 and ASV571) were also recovered from control samples, although at a markedly lower relative abundance. Four ASVs identified as *Gymnopilus* (ASV3, ASV4, ASV13, ASV144) (Basidiomycota), *Fusarium*, *Aspergillus*, *Arthrobotrys* and *Scytalidium* ASVs resulted enriched in the treatments (Figure 2C, Supplementary Table S2). On the other hand, *Conocybe*, *Lindtneria* (Basidiomycota) and *Mucor* (Mucoromycota) were the most depleted ASVs (Figure 2C, Supplementary Table S2).

In order to explore the microbial interactions across the treatments in the rhizosphere, co-occurrence network analysis was performed considering the inter-kingdom interactions. The control and treatment networks consisted of six and seven modules, respectively (Figure 2D,E). The network of control plants showed a nearly balanced distribution between positive and negative interactions, whereas the treatment network was larger and more complex, characterized by a moderate increase in nodes and a significant increase in edges, with the number of positive interactions exceeding the negative ones. The *Trichoderma* ASV84 (ASV84_Fungi) exhibited a distinct interaction profile dominated by negative associations across bacterial and fungal community members (Supplementary Table S3). *Trichoderma* ASV84 showed a relevant negative correlation with the fungus *Actinomortierella* (Mortierellomycota) and bacterial taxa such as *Achromobacter*, *Sphingomonas* (Proteobacteria) and TM7a (Patescibacteria). *Trichoderma* ASV84 showed one strong positive correlation, specifically with the fungal genus *Torula* (Ascomycota) and the bacterial genus *Paenibacillus* (Firmicutes).

3.3. Substantial Restructuring of Phyllosphere Microbial Communities Induced by Bacillus Inoculant

3.3.1. Effect of *Bacillus* Treatment on Phyllosphere Microbial Communities

The foliar application of the *Bacillus*-based formulation significantly altered the bacterial community of the phyllosphere of *C. volkameriana* seedlings. In the phyllosphere, bacterial α-diversity was significantly higher in the control plants than in those treated with *Bacillus* (p = 0.01, FDR adjustment; Figure 3A). A similar trend was observed for fungal communities although the difference was not statistically significant (Kruskal-Wallis test, $p > 0.05$, FDR adjustment; Figure 3B). β-diversity based on Bray-Curtis dissimilarity showed a significant differentiation between treated and control plants for bacterial communities (R^2 “Treatment” = 0.34, PERMANOVA test, $p < 0.05$, FDR-adjusted;

Figure 3C), but not for fungal communities, as assessed by PERMANOVA (R^2 "Treatment" = 0.12, PERMANOVA, $p > 0.05$, FDR-adjusted; Figure 3D).

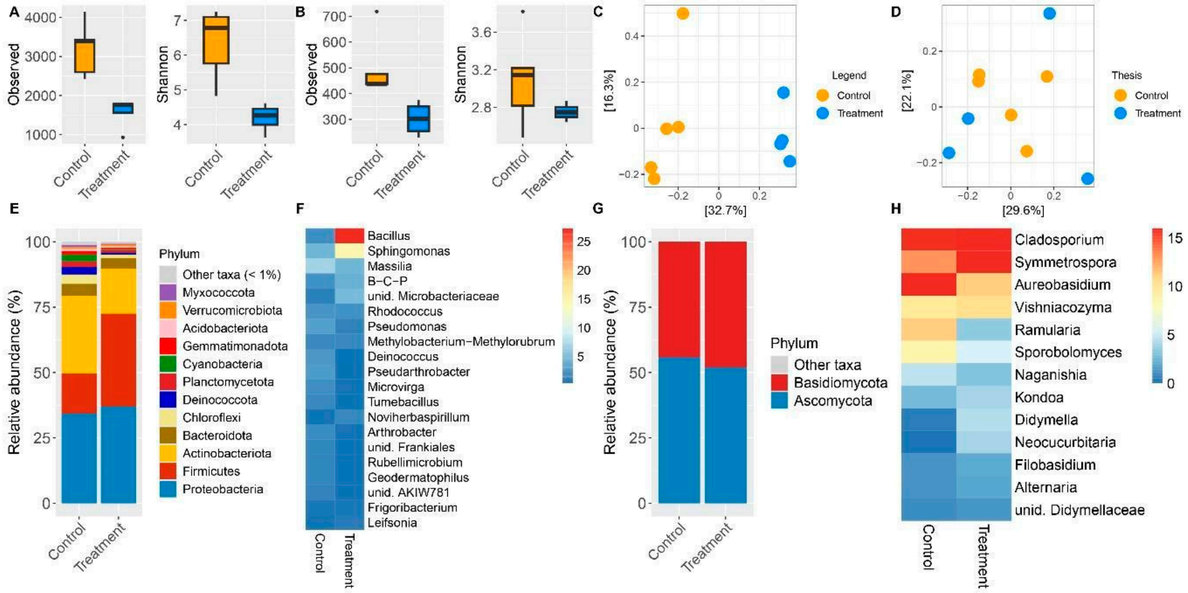

Figure 3. Phyllosphere microbial communities of *Citrus volkameriana* plants treated by leaf spraying with *Bacillus amyloliquefaciens* QST 713 (Serenade® Aso) (Treatment) and untreated plants (Control). α-diversity analysis (Observed and Shannon indices) of the bacterial (**A**) and fungal (**B**) communities. PCoA plot of beta-diversity analysis based on the Bray–Curtis dissimilarity index of bacterial (**C**) and fungal (**D**) communities. Relative abundance of the most abundant bacterial phyla (**E**) and genera (**F**) (>1.5% on the overall) and the most abundant fungal phyla (**G**) and genera (**H**) (>1% on the overall).

The phyllosphere bacterial communities in both control and *Bacillus*- treated plants were predominantly composed of Proteobacteria, Firmicutes, and Actinobacteriota. However, the relative abundance of Proteobacteria (37% vs. 34%) and Firmicutes (35% vs. 15%) was higher in treated plants than in controls, for both phyla, respectively.

The relative abundance of several other phyla was lower in the treated plants as compared to control plants, including Actinobacteriota (17% vs. 29%), Chloroflexi (1.3% vs. 3.6%), Deinococcota (0.72% vs. 2.8%), Planctomycetota (0.9% vs. 2.0%), Cyanobacteria (0.4% vs. 2.7%), and Gemmatimonadota (0.4% vs. 1.2%) (Figure 3E).

The genus *Bacillus* (Firmicutes), the active ingredient of the product applied by foliar spray, became the most abundant genus in treated plants (27%), compared to a background level of only 2% in controls (Figure 3F). *Sphingomonas* (Proteobacteria), the second-most abundant genus in the treated phyllosphere (15%), was also more abundant than in the controls (4%). In the control plants, the genus *Massilia* was a major representative (6%), and there was a more even distribution of over 20 genera accounting for more than 1%, including *Sphingomonas*, *Pseudomonas*, *Bacillus*, *Burkholderia-Caballeronia-Paraburkholderia* (*B-C-P*), *Rhodococcus*, *Deinococcus*, *Pseudarthrobacter*, and *Microvirga* (Figure 3F). The genus *B-C-P* was the third-most abundant in treated plants (4%), with a higher relative abundance than that observed in controls (2%) (Figure 3F).

The phyllosphere fungal communities were dominated by the phyla Ascomycota and Basidiomycota (Figure 3G). The relative abundance of Ascomycota decreased in treated plants (52%) compared to controls (56%), while the abundance of Basidiomycota increased (48% vs. 44%). Fungal community relative abundance at the genus level revealed that *Cladosporium* (Ascomycota) was the most abundant genus in the control and treated plants (15% and 23%, respectively) (Figure 3H). The treatment consistently increased the genera *Symmetrospora* (21% vs. 13%), *Kondoa* (3.9% vs. 2.6%), *Didymella* (4.3% vs. 0.5%), and *Neocucurbitaria* (3.9% vs. 0.2%), whereas it reduced the relative abundance of other common phyllosphere residents, including *Aureobasidium* (11% vs. 21%), *Ramularia* (3% vs. 11%), *Sporobolomyces* (5% vs. 9%), and *Naganishia* (3% vs. 5%).

3.3.2. Modulation of the Phyllosphere Microbial Communities by *Bacillus* Inoculant Application

Bacillus-based formulation affected a substantial restructuring of the phyllosphere bacterial community. Differential abundance analysis identified 23 bacterial ASVs whose abundance was significantly altered by the treatment. The most pronounced effect was the substantial enrichment of a single *Bacillus* ASV (designated as ASV1), which was the predominant taxon within the phyllosphere bacterial communities: the number of reads of ASV1 increased markedly in treated samples, ranging from 34,428 to 43,279, compared to a substantially lower background presence of 9 to 1223 reads in untreated controls (Figure 4A).

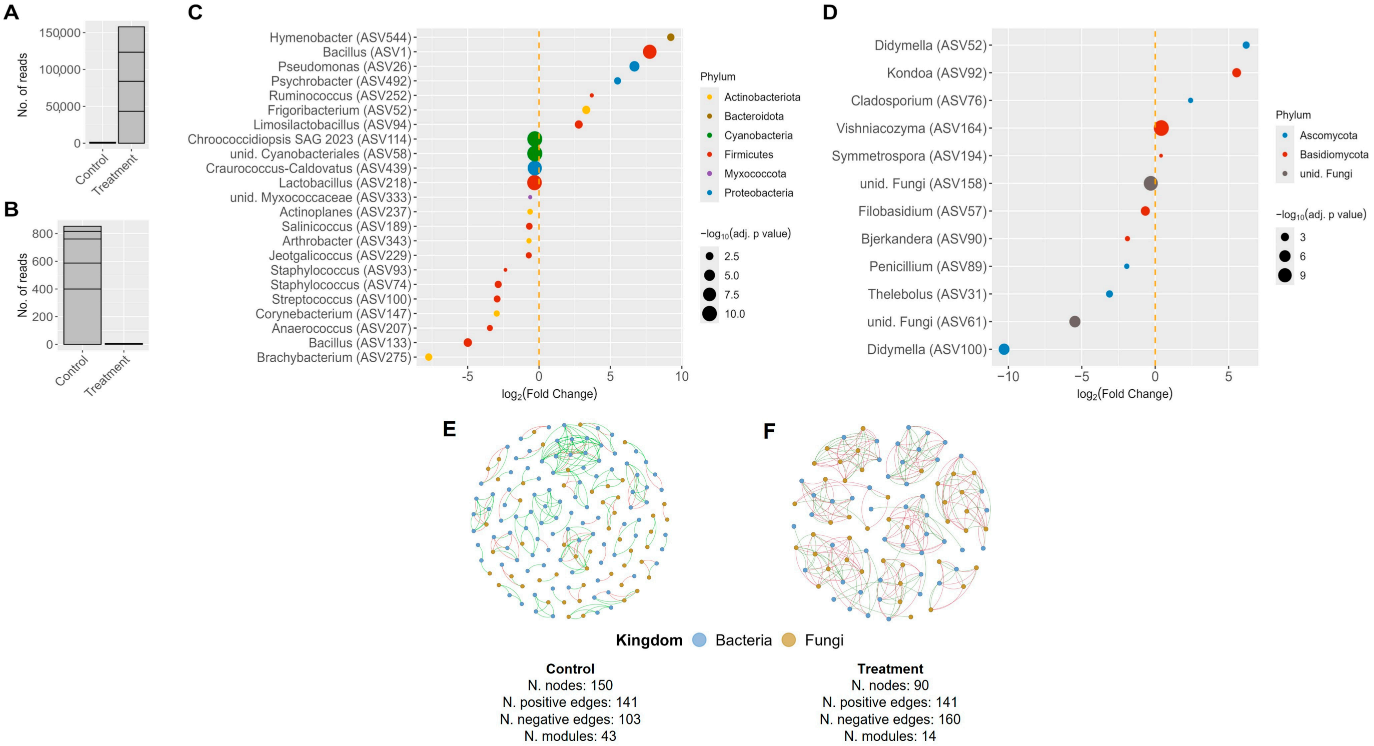

Figure 4. Cumulative absolute abundance of ASV1 (**A**) and ASV133 (**B**) identified as *Bacillus*, significantly enriched and depleted in phyllosphere treated with *Bacillus amyloliquefaciens* QST 713 (Serenade® Aso) (Treatment), respectively. Differentially abundant bacterial (**C**) and fungal (**D**) ASVs of the phyllosphere, statistically significant (–log10 (p) > 1.30, FDR), enriched or depleted, in the treated plants against the untreated ones. Co-occurrence networks of bacterial and fungal ASVs in the control (**E**) and treatment (**F**) samples. Green and red edges represent positive and negative statistically significant interactions ($p < 0.001$), respectively.

The abundance of ASV133 was markedly reduced in treated samples (0–6 reads) relative to controls (55–400 reads) (Figure 4B). *Bacillus* ASV1, enriched in the treated samples, showed 100% identity with different *B. amyloliquefaciens* strains, whereas ASV113 reduced in treated samples showed 94.17% identity with *B. amyloliquefaciens* and 100%

with other *Bacillus* species (e.g., *B. cereus*). Given the divergent abundance patterns and taxonomic profiles, it is inferred that ASV1 corresponds to the applied *B. amyloliquefaciens* inoculant strain, whereas ASV133 likely represents a phylogenetically distinct, indigenous *Bacillus* population that was competitively suppressed.

The other enriched ASVs were assigned to the genera *Pseudomonas*, *Psychrobacter* (Proteobacteria) and *Hymenobacter* (Bacteroidota) (Figure 4C). Conversely, a separate number of bacterial taxa underwent a significant depletion. Another markedly depleted ASVs belong to *Brachybacterium* (Actinobacteriota) and *Anaerococcus* (Firmicutes) (Figure 4C, Supplementary Table S4). Within the fungal community, the genera *Didymella* and *Thelebolus* (Ascomycota) were among the most significantly depleted. Fungal enrichment was assessed for a distinct ASV identified as *Didymella*, alongside *Cladosporium* (Ascomycota) and *Kondoa* (Basidiomycota) (Figure 4D, Supplementary Table S5).

Inter-kingdom co-occurrence network analysis was performed for the phyllosphere compartment, comparing the networks of control and treated plants (Figure 4E,F). In the phyllosphere, the co-occurrence network showed significant changes between control and treatment. The network of control plants was characterized by high modularity, yielding 43 modules compared to 14 in the phyllosphere of treated plants, and exhibited a slight predominance of positive interactions (Figure 4E). Following treatments, the phyllosphere network displayed fewer nodes with a higher number of negative correlations than the ones showed by the network of control plants (Figure 4F).

The *Bacillus* ASV1, referred to as ASV1_Bacteria, interacted with a diverse selection of both bacterial and fungal taxa. In the control network, only negative correlations were detected with two fungal ASVs, represented by *Cladosporium* (Ascomycota) and *Filobasidium* (Basidiomycota), respectively (Supplementary Table S6). In the treatments, *Bacillus* ASV1 showed strong positive correlations with the bacterial genera *Sphingomonas* and *Methylobacterium-Methylorubrum*, and the fungal taxa *Sporobolomyces* (Basidiomycota) and two distinct taxa identified up the Pleosporales order (Ascomycota). *Bacillus* ASV1 also exhibited strong negative correlations with fungal ASVs belonging to the Ascomycota phylum, such as *Ramularia*, multiple ASVs of *Cladosporium* and others identified within the Dothideomycetes class and Pleosporales order (Supplementary Table S7).

4. Discussion

The use of BCAs is a promising strategy for the sustainable management of plant diseases, yet their impact on resident microbial communities remains poorly understood [72,73]. This study employed a metagenomic approach to address this knowledge gap by analyzing the non-target effects of two commercial formulations, one based on *Trichoderma* and the other on *Bacillus*, on the microbiome of the rootstock *C. volkameriana*. This citrus species, susceptible to Mal secco, is one of the most widely used rootstocks in most citrus-producing countries, both for ornamental purposes and fruit production [74,75]. These BCAs have been successfully used to control several plant pathogens [27,28].

Our results showed that the two BCAs exert markedly different influences on the compartment in which they are used according to their application modes. Specifically, soil application of the *Trichoderma*-based product preserved the stability of the rhizosphere microbiome, whereas foliar treatment with *B. amyloliquefaciens* induced substantial restructuring of the phyllosphere bacterial community and maintained the overall richness and evenness (i.e., the uniform distribution of taxa as captured by the Shannon diversity index) within the fungal communities. Comparison of our findings from untreated samples with those of other studies confirms that citrus root microbiome is composed mainly of Proteobacteria [2,13,17,76] and Ascomycota and Basidiomycota [2,17,54] for bacterial and fungal phyla, respectively. In particular, some of the abundant genera found in the rhizosphere were identified in the core citrus rhizosphere microbiome, such as *Paraburkholderia* and *Burkholderia* in the Proteobacteria phylum, and *Rhodococcus* in the Actinobacteria [2].

Application of the formulation containing *T. asperellum* ICC 012 and *T. gamsii* ICC 080 did not significantly alter the α- and β-diversity or overall structure of rhizosphere bacterial and fungal communities, suggesting that it integrates into the root ecosystem without causing large-scale disturbances. Such stability is a desirable attribute since healthy plant microbiome includes key stone taxa within the core microbiome and network of positive interactions. In light of this, maintaining the stability of the microbiome in such plant conditions after BCA applications is of primary importance [77] as a complex and stable soil microbiome can enhance plant resistance to pathogen infections and contribute to overall soil health [78]. However, since changes in the soil microbiome appear to occur more slowly than in the phyllosphere, field trials are needed to examine the rhizosphere for a longer period after *Trichoderma* application [79]. Despite general stability, targeted changes were observed in response to the treatment. First, treatment led to an increase in the relative abundance of the genus *Trichoderma* in treated plants compared to controls, suggesting a successful colonization by the inoculated *Trichoderma* species. Second, differential analysis revealed depletion of a limited number of taxa, including genera such as *Rhodococcus* (Actinomycetaceae) among bacteria, which include both pathogenic and non-pathogenic species [80,81], and *Alternaria* among fungi. This suggests that *Trichoderma* exerts a selective influence rather than a generalized alteration of the community. On the contrary, some bacterial genera resulted enriched in treated samples, including *Pantoea (*Erwiniaceae) and *Paenibacillus* (Firmicutes), which are generally known for their ability to promote plant growth and nutrient mobilization [82–84]. Interestingly, some Saccharimonadales ASVs (Patescibacteria) were enriched in the treated samples. These Patescibacteria are not deeply understood for their behavior, but they are presumed to have a symbiotic or parasitic lifestyle, often reported in the rhizosphere for the presence of high sugar root exudated content and high P availability [85–87]. Moreover, some fungal taxa were differentially abundant in *Trichoderma*-treated samples, although these changes were not significantly different, such as *Gymnopilus*, known for its role in organic matter degradation [88], unid. Mortierellaceae, which includes important species known as plant growth-promoting fungi (PGPF) often found in the extremely hostile environments, responsible for improving plant uptake to the bioavailable forms of P and Fe in the soils, the synthesis of phytohormones, and the protection of plants from pathogens [89], and *Arthrobotrys*, which contain species with ability to prey on nematodes [90]. Similar results were observed in other studies investigating the effects of *Trichoderma* on microbiome [91,92], which reported that *Trichoderma* soil application maintains the stability and complexity of the rhizosphere microbial network. This stability may enhance the resistance of the plant to pathogen infection and play also a crucial role in soil ecology and sustainable agricultural development [28,93,94].

Analyses of inter-kingdom co-occurrence networks in the rhizosphere compartment revealed that treatment with *Trichoderma* deeply modified the network structure, resulting in a higher number of edges, especially negative, compared to the control. These negative interactions are consistent with the ecological role of many *Trichoderma* species as mycoparasites and aggressive competitors in the rhizosphere [95]. Interestingly, *Trichoderma* ASV84, identified as *T. asperellum* with a 100% of identity, showed one strong positive correlation with an ASV identified as *Paenibacillus*. This interaction is biologically meaningful, as *Paenibacillus* species are often recognized as plant-growth-promoting rhizobacteria [76,96] and have been shown in other studies to interact synergistically with *Trichoderma,* potentially contributing to enhance plant health and pathogen suppression in the root environment [97]. A negative correlation was also observed between *Trichoderma* ASV84 and a bacterial ASV assigned to *Sphingomonas*, a bacterial genus detected in multiple citrus compartments [12] and recognized as a potential PGPR [98]. For this reason, the strong ability of *Trichoderma* to compete with or establish antagonistic interactions with other microorganisms could represent a disadvantage if it interferes with the PGPR activity of beneficial bacteria such as *Sphingomonas*.

Concerning the samples obtained from the *Bacillus*-treated phyllosphere, differences in enriched phyla between control and treated samples were observed. The phyllosphere collected from control plants were composed for 90% of Proteobacteria, Firmicutes, Actinobacteriota, Bacteroidota, Chloroflexi and Deinococcota, whereas in treated plants only Proteobacteria, Firmicutes, Actinobacteriota reached the 90%. These results are consistent with existing knowledge of the microbiomes of citrus phyllosphere and carposphere [14,54,99,100], who also found that the ratio between these phyla varies significantly according to citrus varieties, environmental conditions, and plant health conditions. Therefore, foliar application of *B. amyloliquefaciens* QST 713 strongly reshaped the phyllosphere bacterial microbiome. A significant reduction in bacterial α-diversity was consistently observed in treated plants compared to controls, accompanied by clear differentiation in community composition, as shown by the β-diversity analysis. Studies evaluating the effects of application of beneficial bacteria individually or as synthetic community on the phyllosphere microbiome of citrus [53,55] reported similar results. Specifically, *Pseudomonas parafulva* SAPEU-1 [55], and *Paenibacillus* sp. ATY16, *B. megaterium* PT6, *B. subtilis* PT26A and SynCom1 363 (ATY16 + PT6 + PT26A) [53] significantly reshape the citrus leaf microbiome resulting in a significantly different leaf bacterial community diversity and structures compared to the untreated control. In our study, this shift was driven by successful colonization by the biocontrol agent, with the genus *Bacillus* becoming the most abundant in the treated phyllosphere. Notably, beyond *Bacillus* dominance, the treatment promoted enrichment of other genera known for beneficial effect on plant growth, such as *Pseudomonas*, *Methylobacterium*, *Sphingomonas*, and *Burkholderia-Caballeronia-Paraburkholderia* group. Similarly, Li et al. [53] reported that ASVs belonging to *Bacillus*, *Paenibacillus* and *Burkholderia* were enriched after inoculation with SynCom1 363, and Asad et al. [55] observed a high level of *Sphingomonas, Methylobacterium,* and *Rhizobium* after *Pseudomonas parafulva* SAPEU-1 treatment. This suggests that beneficial bacteria, such as *B. amyloliquefaciens* tested in our study, modulates the phyllosphere toward a new community composition that may play an additional role in disease suppression. Regarding fungal communities, the phyllosphere was dominated by the phyla Ascomycota and Basidiomycota, and the *Bacillus* treatments induced a slight but not significant shift in their balance. Although the differential abundance analysis showed that some fungal ASVs identified up the genus level were significantly enriched (e.g., *Symmetrospora*, *Didymella* and *Neocucurbitaria*) or depleted (e.g., *Aureobasidium*, *Ramularia*, *Sporobolomyces* and *Naganishia*), *Bacillus* treatments maintained the overall richness/evenness.

The inter-kingdom co-occurrence network analysis was also performed on the phyllosphere microbial communities and a different network structure between control and treated specimens was observed. The treatment with *Bacillus* affected microbial interactions compared to the control conditions, causing a reduction in network modularity and an increase in negative correlations. This result is consistent with the effects of biocontrol agents in the phyllosphere of other plant models [101] and is also similar to other studies reporting a decrease in the modularity of phyllosphere microbial networks after plant pathogen inoculation [60]. The representative ASV1 of *Bacillus* exhibited strong positive correlations with bacterial ASVs identified as *Sphingomonas* and *Methylobacterium-Methylorubrum*, whose roles as biocontrol agents and ecological cooperation in the phyllosphere of other plant systems have already been documented [102]. Although the plant growth-promoting functions of these genera have been established in citrus microbiomes under biotic stress from *Diaporthe citri* [60] or *Pseudomonas syringae* [103], significant correlations with *Bacillus* have not been documented in those studies, in contrast with our observations. Interestingly, *Bacillus* ASV1 was negatively correlated with *Ramularia* and *Cladosporium*: these fungal genera comprise different species with several roles, including potential phytopathogens in citrus [104–106]. A positive correlation between the *Bacillus* ASV1 and an *Alternaria* ASV was observed. This fungal genus includes several species with different roles in citrus plants [107] and it is well known that *B. amyloliquefaciens* has a BCA role against *Alternaria* pathogens in several host plants [108,109]. However, although the genus *Alternaria* includes many pathogenic species in citrus [4], this apparent inconsistency can be explained by the prevalence of non-pathogenic *Alternaria* taxa on the citrus plants which maintain an ecological balance and prevent the increase in pathogenic species. Interestingly, some of the genera found in high level in our study are reported as core members of the citrus phyllosphere microbiome, including *Bacillus*, *Pseudomonas*, *Massilia*, *Methylobacterium*, *Sphingomonas*, and *Alternaria* [4,8].

High sequence similarity to the inoculated *Bacillus* and *Trichoderma* species was found through BLAST search of the ASVs enriched in the BCA-treated samples. This suggests that these taxa, which likely represent the inoculated strains, successfully established within the specific compartment in which they were applied due to the different intrinsic ecological traits of each BCA. *Trichoderma* spp., in fact, are soil-born fungi acting through a variety of mechanisms, including root colonization, nutrient solubilization and bioavailability in the soil, abiotic and biotic stress tolerance, secondary metabolite production, and the synthesis of plant hormones such as auxins, gibberellins, and cytokinins [28]. On the contrary, *Bacillus* species are natural inhabitants of both rhizosphere and phyllosphere considered plant beneficials since they produce endospores and metabolites with antimicrobial or plant growth promotion activity, such as antibiotics, enzymes, vitamins and secondary metabolites [24]. The efficacy of BCAs tested in this study were already demonstrated against *P. tracheiphilus* on *C. volkameriana*, a citrus species selected as a model plant for its high susceptibility to Mal secco [42–44,76]. *Trichoderma asperellum* ICC 012 and *T. gamsii* ICC 080 formulate was effective in reducing foliar infections of *P. tracheiphilus* when applied as root drench suggesting that its effectiveness can be attributed also to indirect activity that involve plant induction of systemic resistance (ISR), that primes plants to respond more quickly and strongly to pathogen attacks [44,110]. Foliar application of *B. amyloliquefaciens* QST 713 also reduces the disease incidence and symptom severity in Volkamer lemon seedlings inoculated with *P. tracheiphilus* [42]. Our findings suggest that the protective efficacy of *B. amyloliquefaciens* may derive not only from direct competition and antimicrobial production, but also through microbiome modulation. However, findings of our study should be interpreted taking into account the distinct microenvironmental conditions of the plant-compartments analyzed (e.g., phyllosphere and rhizosphere). In general, the diversity and stability of the rhizosphere citrus microbiomes are higher than that of the phyllosphere microbiome, since the roots are protected by soil that provides a more stable environment less affected by weather conditions and agricultural practices, including BCA applications, than the above-ground plant compartment [77,111].

In conclusion, findings of this study suggest that the application of these BCAs, and particularly of *Bacillus*, may have an effect that goes beyond the simple interaction with the target pathogen, instead modulating the host's microbial ecology and improving the plant health. Since our study only focused on the impact of BCAs on two separate niches (phyllosphere and rhizosphere) without assessing their effects on plant growth, investigations aimed at clarifying whether *Trichoderma* and *Bacillus* are involved in other plant-beneficial effects, such as improving yields, tolerance to abiotic stress and increase in nutrient uptake, are needed. Furthermore, while this nursery study provides a first screening, we acknowledge the limitation in predicting impact of BCAs on rhizosphere and phyllosphere microbiome under field conditions. For this reason, further investigations are required to clarify their sustainability under field conditions, as well as to better understand how these BCAs interact with plant microbiome in terms of affecting the microbial fitness, population dynamics and functional ability. It is important to highlight that the co-occurrence analysis may be useful to identify potential hub species and give an idea about the complexity of microbial interaction networks, including those influenced by BCA applications. However, they are not enough to unravel the nature of BCA-microbiome interactions and the metabolic patterns.

5. Conclusions

This study provides a first assessment of the response of citrus-associated bacterial and fungal communities to two major commercial BCAs reported to be effective against fungal citrus pathogens in Italy [42–45]. By using high-throughput sequencing methods, this study ascertained the ability of *Trichoderma*-based formulation to promote soil stability and the capacity of *Bacillus*-based formulation to enrich beneficial bacterial populations known as PGPR in Volkamer lemon plants. These findings make BCAs such as *B. amyloliquefaciens* QST 713 and *T. asperellum* ICC 012 + *T. gamsii* ICC 080 promising candidates as a sustainable alternative to copper-based fungicides, whose use is increasingly restricted and disturbance on microbiome was reported [54]. Furthermore, previous studies reported *Trichoderma* and *Bacillus* enhancing citrus growth by increasing nutrient availability and promoting plant hormone production, as well as reducing plant abiotic stress [112–114]. For these reasons, future research should explore and integrate complex three-way interactions (BCA-pathogen-microbiome) with agronomic evaluation under field conditions to develop more effective and predictable biocontrol strategies for managing citrus Mal secco disease. This would permit a deeper understanding of the role of these BCAs on plant growth and disease management.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae12050529/s1, Table S1: Differentially abundant bacterial ASVs of the rhizosphere, enriched or depleted and statistically significant ($p < 0.05$, FDR), in the treated plants against the untreated ones; Table S2: Differentially abundant fungal ASVs of the rhizosphere, enriched or depleted and statistically significant ($p < 0.05$, FDR), in the treated plants against the untreated ones; Table S3: Differentially abundant bacterial ASVs of the phyllosphere, statistically significant ($p < 0.05$, FDR), enriched or depleted, in the treated plants against the untreated ones. Table S4: Differentially abundant fungal ASVs of the phyllosphere, enriched or depleted, and statistically significant ($p < 0.05$, FDR) in the treated plants against the untreated ones. Table S5: Statistically significant interactions between *Trichoderma* ASV84 and other bacterial and fungal ASVs in the rhizosphere of treated plants. Table S6. Statistically significant interactions between *Bacillus* ASV1 and other bacterial and fungal ASVs in the phyllosphere of no-treated plants. Table S7. Statistically significant interactions between *Bacillus* ASV1 and other bacterial and fungal ASVs in the phyllosphere of treated plants.

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Conflicts of Interest: The authors declare no conflicts of interest.

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