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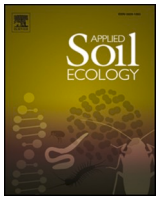

Brevibacterium EB3 inoculation enhances rhizobacterial community interactions leading to improved growth of *Salicornia europaea*

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ABSTRACT

Plant growth-promoting bacteria (PGPB) can revolutionize sustainable agriculture by improving crop yields and resilience in the face of climate change and soil degradation. However, one of the challenges of using PGPB is identifying strains that can colonize and establish beneficial relationships with plant hosts and microbiomes. This study examined the effects of single and co-inoculations with three PGPB strains (*Brevibacterium casei EB3*, *Pseudomonas oryzihabitans* RL18, and *Bacillus aryabhattai* SP20) on the rhizosphere microbiome of the halophyte crop *Salicornia europaea*. 16S rRNA gene amplicon sequencing was used to analyze the rhizosphere bacterial communities' diversity, structure, and composition. PGPB inoculations significantly changed the diversity and structure of the bacterial communities in the rhizosphere, accounting for 74 % of the total variability. The strain *B. casei* EB3 was the most effective at colonizing the rhizosphere and establishing interactions with other beneficial community members. Notably, the treatments associated with higher plant yield, consistently featured the presence of *B. casei* EB3 and higher connectivity between this strain and taxa known to promote growth and alleviate salt stress in plants such as *Marinobacterium*, *Pseudomonas* and *Vibrio*. These findings are consistent with bacterial inoculants' direct and indirect effect in boosting bacteria-plant cooperation within the rhizosphere, ultimately leading to a shift towards an optimized rhizosphere and beneficial traits for plants.

1. Introduction

The rhizosphere, the soil zone surrounding plant roots, is a hot spot for microbial activity and plant-microbe interactions (Reinhold-Hurek et al., 2015). Root-associated bacterial communities (rhizobacteria) can enhance plant growth directly or indirectly by different mechanisms. Their strategies involve mobilizing nutrients and minerals from the soil (Rengel, 2015), suppressing microbial pathogens (Santhanam et al., 2015) and herbivory (Hubbard et al., 2019), enhancing plant immunity and eliciting defensive responses by modulating phytohormone levels (Finkel et al., 2020) and increasing tolerance to abiotic stress, like drought (Vejan et al., 2016) and salinity (Santos et al., 2021). Collectively referred to as plant growth-promoting bacteria (PGPB), these beneficial bacteria are a promising biotechnological approach for sustainable agriculture (Chandran et al., 2021).

Halophytes can tolerate salt concentrations that would kill 99 % of other plants (Flowers and Colmer, 2008) by developing physiological and biochemical adaptations to saline environments and establishing adequate symbiotic associations with microorganisms (Etesami and Beattie, 2018). The coastal halophyte *Salicornia europaea* is an excellent resource for PGPB (Ferreira et al., 2021). Several PGPBs isolated from the rhizosphere of halophytic species have demonstrated their potential application as bio-inoculants to promote growth and enhance the salinity tolerance for the sustainable crop production of non-halophyte and halophyte plants (Etesami and Beattie, 2018).

Recently, special attention has been given to bacterial consortia, which combine multiple strains of bacteria to obtain a desirable set of plant growth-promoting traits. These consortia are expected to have greater synergies than single-strain inoculations, leading to more favorable outcomes (Mesa-Marín et al., 2019; Santoyo et al., 2021). The communities of plant microbiota are dynamic, complex, and highly diverse. Therefore, it has been speculated that the beneficial traits rely on microbial cooperation and the functions of the entire microbiome, not only on an individual microorganism (Vandenkoornhuyse et al.,

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2015). Supporting this hypothesis, some studies have shown that mixtures of microorganisms rather than individual microbial species can strengthen beneficial effects on crops such as drought stress attenuation in rice (Joshi et al., 2020), wheat growth and yield under saline conditions (Nawaz et al., 2020), and nutrient uptake (Rana et al., 2012). Although co-inoculation seems a promising strategy for improving plant growth and yields, more research is needed to understand how single inoculations and co-inoculations affect the plant microbiome and how these changes can be exploited to improve crop production.

Ferreira et al. (2023) tested different combinations of selected PGPB in single inoculations and co-inoculations on the growth of *S. europaea* in the laboratory and field conditions. The authors reported that plants inoculated with a specific combination of two PGPB strains in controlled laboratory conditions had the highest increase in biomass, which was explained by increased amino acid biosynthesis. Although the detection of the bacterial inoculants in the grown plants was reported, the authors did not investigate the impact of inoculations on the resident plant microbiome. As the effectiveness of PGPB inoculation depends on their ability to colonize the rhizosphere and interact with other microbes, we investigated the impact of the single and co-inoculations of PGPB strains on the rhizosphere of *S. europaea* under controlled conditions. We hypothesize that inoculating different combinations of beneficial PGPB will alter the rhizosphere microbiome in different ways, which may potentiate or mask the beneficial effects of the individual bacteria on plant growth. Specifically, we are interested in studying key microbial members and their synergistic interactions. Understanding the direct and indirect effects of PGPB inoculation on the rhizosphere microbiome is essential for developing rhizosphere engineering programs that can reliably predict improvements in plant growth under sustainable agriculture practices.

2. Materials and methods

2.1. Inoculation with PGPB and plant growth in S. europaea

Three bacterial inoculants from the culture collection of the Laboratory of Environmental Microbiology (LMICRA) of University of Aveiro were selected based on their plant-growth promotion traits (Ferreira et al., 2021): *Brevibacterium casei* EB3 has nitrogen fixation activity and produces 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase and indole acetic acid (IAA); *Pseudomonas oryzihabitans* RL18, can solubilize phosphate, produce siderophores, ACC deaminase and IAA; *Bacillus aryabhattai* SP20, is a prolific enzyme producer capable of solubilizing phosphate and producing siderophores. These bacteria were used in inoculation and co-inoculation essays in seeds of *S. europaea*. The experimental design and the method for the *S. europaea* seed inoculation are described in Ferreira et al. (2023). Briefly, sterilized seeds (1:1 mixture of hydrogen peroxide and ethanol) were inoculated with single or combined bacterial inoculants, as follows: 1) EB3: sole inoculation with *B. casei* EB3; 2) RL18: sole inoculation with *P. oryzihabitans* RL18; 3) SP20: sole inoculation with *B. aryabhattai* SP20; 4) EB3 + RL18: co-inoculation with *B. casei* EB3 and *P. oryzihabitans* RL18; 5) EB3 + SP20: co-inoculation with *B. casei* EB3 and *B. aryabhattai* SP20; 6) RL18 + SP20: co-inoculation with *P. oryzihabitans* RL18 and *B. aryabhattai* SP20; 7) ALL: co-inoculation with *B. casei* EB3, *P. oryzihabitans* RL18 and *B. aryabhattai* SP20; 8) CONTROL: non-inoculated control (Table 1).

Bacterial inoculants were grown separately in Tryptic Soy Broth (Liofilchem, 25 g L^{-1} NaCl) at 150 rpm and 30 °C. Cells were collected, washed twice with sterile saline solution (9 g L^{-1}NaCl) and re-suspended in sterile saline solution to a concentration of 10^8 CFU mL^{-1} (OD600 ~ 1) (Mesa-Marín et al., 2019). *S. europaea* seeds were collected from a crop cultivation facility in autumn (November 2020) (Horta dos Peixinhos, Aveiro, Portugal). Surface-sterilized seeds were inoculated by adding the appropriate bacterial suspension and incubating for 2 h. For combined inoculations, equal volumes of the bacterial suspensions were mixed (1:1). Non-inoculated control seeds were exposed to a sterile saline solution. Inoculated seeds were germinated in 1 % agar plates and then transferred to plastic pots filled with perlite permeabilized with 20 % Hoagland's solution. The pots were maintained in an outdoor greenhouse under natural sunlight. After two months, plants were transferred to larger pots filled with perlite and sterilized salt marsh sediment (1:1 ratio). Plants were watered every two days using 20 % Hoagland's solution containing 10 g L^{-1} marine salt. Every 15 days, the plants were re-inoculated with the same inoculation treatment directly on the soil. Non-inoculated controls received an equivalent volume of sterile water. The plants were maintained in the outdoor greenhouse for additional 30 days. To prevent adverse effects associated with premature aging and senescence, the plants were transferred to a climate chamber (Fitoclima D1200, Aralab, Sintra, Portugal) and kept under controlled conditions: 16/8 h photoperiod, 25/20 °C temperature, 40 % relative humidity, and 500 μmol m^{-2} s^{-1} photon flux density. The effects of the inoculation treatments on plant growth were determined at the end of the experiment (approximately five months from the inoculation of seeds until the harvesting of plants). In addition, further detection of the bacterial inoculants in the plants is summarized in Table 1.

2.2. Rhizosphere sampling

Three replicate individuals (plants) from each experimental condition were used to analyze rhizosphere microbial communities. To collect rhizosphere material, unwashed plants were hand-shaken to remove loosely attached bulk soil. Roots with attached soil were separated for the aerial part with sterile scissors and placed into sterile 50 mL conical tubes. Each tube received 35 mL of sterile phosphate-buffered saline buffer (PBS) supplemented with 0.01 % Tween 80 and was vortexed for

Table 1
Summary of the plant growth effect and detection of the PGPB strains inoculated in *S. europaea* plants in each inoculation treatment reported in Ferreira et al. (2023).

Inoculation treatment	Inoculated strain(s)	Increased factor of growth stimulation in relation to non-inoculated control	Presence (+) or absence (−) of ASVs in the *S. europaea* rhizosphere matching 100 % the inoculant strain		
			Brevibacterium casei EB3	*Pseudomonas oryzihabitans* RL18	*Bacillus aryabhattai* SP20
EB3	*Brevibacterium casei* EB3	2,2	+	−	+
RL18	*Pseudomonas oryzihabitans* RL18	•	−	−	−
SP20	*Bacillus aryabhattai* SP20	•	−	−	+
EB3 + RL18	*B. casei* EB3 and *P. oryzihabitans* RL18	4,6	+	−	−
EB3 + SP20	*B. casei EB3* and *B. aryabhattai* SP20	•	+	−	−
RL18 + SP20	*P. oryzihabitans* RL18 and *B. aryabhattai* SP20	•	−	−	−
ALL	*B. casei* EB3, *P. oryzihabitans* RL18 and *B. aryabhattai* SP20	3,9	+	−	−
CONTROL	Non-inoculated	•	−	−	−

• Non-significant increase plant biomass compared to control plants.

5 min (Barillot et al., 2013). Root material was removed and rinsed several times until the root surfaces were clear. The rinsing liquid was added to the tube. Rhizosphere material, collected by centrifugation of the soil suspension at 10000 *g*, was deep-frozen (−80 °C) until DNA extraction.

2.3. DNA extraction and sequencing

DNA extraction was performed using FastDNA™ SPIN Kit for soil (MP Biomedicals, France). The V3-V4 hypervariable region of the 16S rDNA gene was amplified using primers Bakt_341F 5′–CCTACGGGNGG CWGCAG-3′ and Bakt_805R 5′–GACTACHVGGGTATCTAATCC-3′ (Herlemann et al., 2011; Klindworth et al., 2013). Negative PCR controls were included for all amplification procedures. PCR products were purified and normalized using a SequalPrep 96-well plate kit (ThermoFisher Scientific, Waltham, USA) (Comeau et al., 2017) and pair-end sequenced in the Illumina MiSeq® platform with the MiSeq reagent Kit v3 (600 cycles), according to manufacturer's instructions (Illumina, Sand Diego, CA, USA) at Genoinseq (Cantanhede, Portugal). Sequences used in this study have been uploaded to the NCBI Short Read Archive under the BioProject number: PRJNA858439.

2.4. Bioinformatic analysis

Raw reads were extracted from Illumina MiSeq® System in fastq format. Further bioinformatic analyses were processed and analyzed using Quantitative Insights into Microbial Ecology QIIME2 (Bolyen et al., 2019) package version 2020.8 and its plugins. The DADA2 plugin (Callahan et al., 2016) was used to trim sequences, removal of chimeras (consensus method) and grouping into representative sequences called amplicon sequence variants (ASVs). Taxonomy was assigned to ASVs using q2-feature-classifier plugin (Bokulich et al., 2018) against the Silva138 99 % OTUs reference sequence (Quast et al., 2013) (available at https://docs.qiime2.org/2019.4/ in June 2021). Downstream analyses were performed on R v4.0 (R Core Team, 2014) and the Phyloseq package (McMurdie and Holmes, 2013). All non-bacterial ASVs were removed. Alpha diversity indexes (observed, Shannon, Chao and Simpson) were calculated by normalizing the sequence number to the minimum sample size (21,784) by random subsampling. One-way ANOVA with the inoculation treatment as factor was performed using the R function aov(), followed by a pairwise *t*-test to determine which inoculant contributed the most to the variation of alpha diversity data compared to the control. For Beta diversity, amplicon sequencing data were normalized using DESeq2 (Love et al., 2014). Principal Coordinate Analysis was employed on Bray Curtis distance matrix using the "ordinate" function in the Phyloseq package; significantly different clusters were determined using "adonis" with the "betadisper" test to check for equal variance in vegan package. The detection of the inoculants was performed as in Ferreira et al. (2023) using the 16S rRNA sequence of the isolates EB3, RL18 and SP20 in a blast alignment against all the ASVs in the dataset. Two ASVs matched 100 % with the sequence of *B. casei* EB3 and one ASV with *B. aryabhattai*. In contrast, any ASV in the data set matched 100 % the sequence of *P. oryzihabitans* RL18 at 100 %. Here, we used the most similar ASVs that matched *P. oryzihabitans* RL18 for phylogenetic analysis to confirm the identification of the sequence in MEGAX (Tamura et al., 2021). In addition, the number of ASVs assigned to the genera *Pseudomonas* sp., *Bacillus* sp. and *Brevibacterium* sp. were extracted from the datasets. Differential abundant taxa between the inoculation treatments and the non-inoculated control were investigated using DESeq2-phyloseq (Love et al., 2014). In addition, SIMPER test was used as an additional method to determine the ASVs that most contributed to the total dissimilarity in the same pairwise comparisons between the inoculation and the non-inoculated control. We only kept significant differential bacterial taxa ($p < 0.001$) found in abundances higher than 0.03 %, which showed higher than 0.2 % SIMPER contribution to the total dissimilarity between pairwise comparisons. For co-occurrence network analysis, bacterial ASVs were filtered to remove those that did not occur in at least 2 of the replicates for each sample treatment. Then, SPIEC-EASI networks were constructed using the spiec.easi function of the SPIEC-EASI library (Kurtz et al., 2015). The resulting object (a sparse adjacency matrix) was used to build a microbiome network for each treatment. We identified hubs using centrality indices and listed network features for each treatment as described by Layeghifard et al. (2018).

3. Results

3.1. Diversity of rhizosphere bacterial communities in inoculated and non-inoculated plants

Triplicate amplicon sequences from the V3-V4 region of the 16S rRNA gene were obtained from the rhizosphere of plants belonging to 8 inoculation treatments (24 samples). A total of 1,899,539 raw sequences (median 77,712 sequences per sample) were obtained. Filtering, denoising and removal of chimeras resulted in 731,040 high-quality sequences (SI Table S1). The number of sequences ranged from 21,784 to 49,352 sequences per sample. After removing sequences not classified as bacteria (chloroplasts, mitochondria, archaea, unassigned), 2661 different ASVs were obtained with an average of 730 ASVs per sample (SI Table S1). All samples were subsequently rarified to 21,784 sequences for alpha diversity analysis. The rarefaction curve analysis showed that the subsampling of sequences still yielded a sufficient resolution of bacterial communities (SI Fig. S1). The treatment with all the inoculants (ALL) accounted for the lowest number of observed ASVs, while the inoculation with *B. casei* EB3 accounted for the highest number of ASVs (SI Fig. S2, SI Table S2).

Alpha diversity measures are presented in SI Table S2. The rhizosphere microbiome inoculated with *B. casei* EB3 exhibited the highest observed ASVs (Fig. 1A). One-way ANOVA revealed a more substantial significant effect ($p < 0.005$) of the inoculation treatment on rhizobacterial species richness (represented by observed ASVs) than on the Shannon index (SI Table S3-S4). However, pairwise comparisons showed that only the inoculation with *B. casei* EB3 caused a statistically significant increase in observed ASVs compared to the control ($p < 0.005$, Fig. 1A). Fig. 1B represents the beta diversity, measured by Bray Curtis distances for the inoculation treatments. The PCoA analysis of rhizobacterial communities revealed that ALL and RL18 + SP20 and non-inoculated (CONTROL) treatments were separated from all single-strain inoculations and co-inoculations EB3 + RL18 and EB3 + SP20 along the first principal coordinate axis (35.7 % of the variability) (Fig. 1B). The second coordinate axis (10.2 % of the variability) differentiated the single inoculation with *P. oryzihabitans* RL18 from all other experimental conditions. Rhizobacterial community structure was significantly influenced by inoculation, whether with single strains or mixtures, accounting for 74 % of the variance according to Permutational Analysis of Variance (PERMANOVA) (SI Table S5). Notably, the treatment RL18 + SP20 contributed the most to the variance (20 %, $p = 0.001$), followed by the combination of EB3 + RL18 (11 %, $p = 0.001$). Among the single inoculations, EB3 and RL18 had the most significant effects, accounting for 10 % and 8 % of the variance ($p = 0.001$), respectively (SI Table S5).

3.2. Taxonomic composition of rhizobacterial communities

Seventy-nine bacterial classes were detected, but only 18 were considered dominant, with abundances higher than 1 % (Fig. 2). In general, the rhizobacterial communities were predominantly composed of the following taxonomic classes and their corresponding average abundance Alphaproteobacteria (16 %), Gammaproteobacteria (16 %), Bacteroidia (16 %), Planctomycetes (9 %), Verrumicrobiae (6 %), Rhodothermia (5 %), Actinobacteria (3 %) and Anaerolineae (3 %). Some taxa were more associated with particular inoculation conditions.

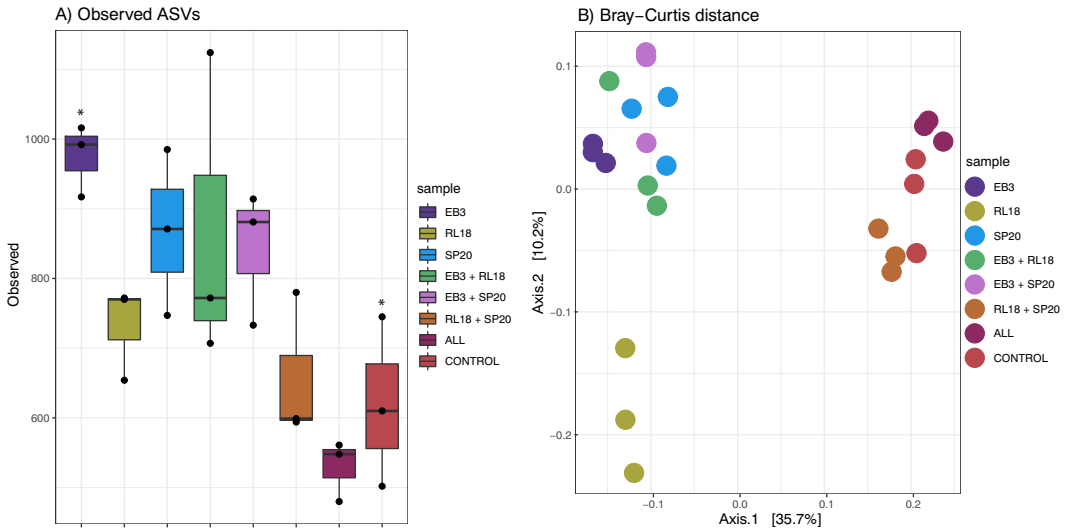

Fig. 1. Bacterial diversity found in *S. europaea* rhizosphere exposed to different inoculated conditions. (A) Alpha diversity measured by observed ASVs. (B) PCoA plot of rhizobacterial composition based on Bray-Curtis distance for the inoculated treatments. CONTROL: non-inoculated; EB3: *Brevibacterium casei* EB3; RL18: *Pseudomonas oryzihabitans* RL18; SP20: *Bacillus aryabhattai* SP20; EB3 + RL18: *B. casei* EB3 and *P. oryzihabitans* RL18; EB3 + SP20: *B. casei EB3* and *B. aryabhattai* SP20; RL18 + SP20: *P. oryzihabitans* RL18 and *B. aryabhattai* SP20; ALL: *B. casei* EB3, *P. oryzihabitans* RL18 and *B. aryabhattai* SP20. * Significant t-test ($p < 0.005$).

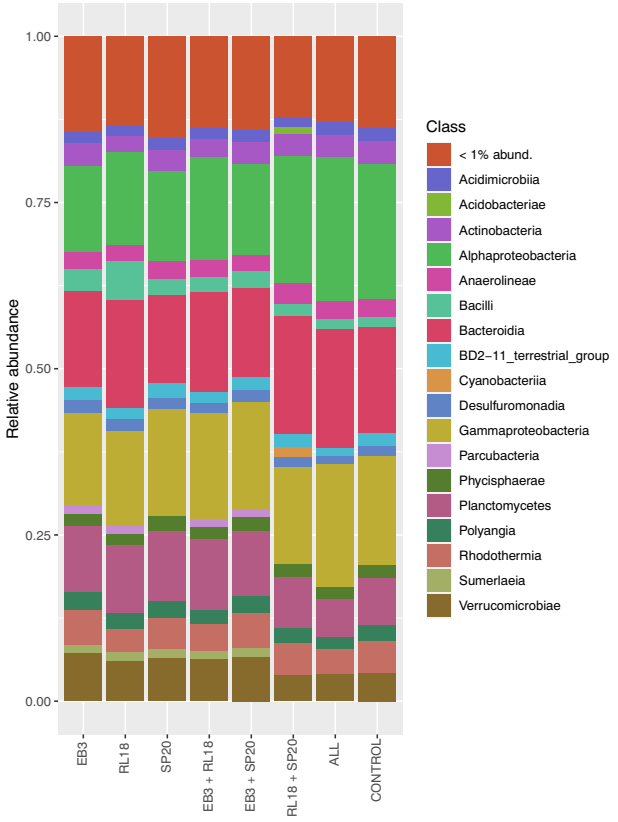

Fig. 2. Relative abundance of dominant (<1 %) of bacterial taxa, at the class level, in the rhizosphere of *S. europaea* corresponding to different inoculation treatments. EB3: *Brevibacterium casei* EB3; RL18: *Pseudomonas oryzihabitans* RL18; SP20: *Bacillus aryabhattai* SP20; EB3 + RL18: *B. casei* EB3 and *P. oryzihabitans* RL18; EB3 + SP20: *B. casei EB3* and *B. aryabhattai* SP20; RL18 + SP20: *P. oryzihabitans* RL18 and *B. aryabhattai* SP20; ALL: *B. casei* EB3, *P. oryzihabitans* RL18 and *B. aryabhattai* SP20; CONTROL: non-inoculated.

Acidobacteriae and Cyanobacteriia showed relative abundances higher than 1 % in the RL18 + SP20 treatment. Parcubacteria was more represented in treatments EB3, RL18, EB3 + RL18 and EB3 + SP20. Candidatus Sumerlaeia was dominant (>1 %) in almost all the inoculation treatments except for RL18 + SP20, ALL and CONTROL.

Considering the top 50 ASVs (log abundance) across all inoculation treatments (Fig. 3), the treatment involving the inoculation of ALL the bacterial strains and the non-inoculated CONTROL displayed a similar pattern. This pattern was characterized by the detection of higher abundances of specific ASVs assigned to *Vibrio* and the absence of an ASV assigned to *Pseudomonas*. In contrast, the single inoculation with EB3 exhibited higher frequencies of ASV belonging to Gimesiaceae and *Tistrella*, while several ASVs assigned to *Vibrio, Exiguobacterium* sp. and Oscillatoriaceae were absent. The pattern observed for the single inoculation with RL18 closely resembled the pattern depicted at the inoculation with EB3. Notably, the inoculation with EB3 + RL18 was distinctly characterized by the presence of two specific ASVs assigned to *Marinobacterium* and *Vibrio* sp., along with a high abundance of *Pseudomonas* and *Haloferula*. A higher abundance of *Tistrella*, Gimesiaceae, *Exiguobacterium* and other *Vibrio* sps characterized all the other treatments.

3.3. Further detection of PGPB inoculants on the rhizosphere community

Although previously reported (Table 1), the bacterium *B. casei* EB3 was detected in the inoculation treatments where it was added. The bacterium *B. aryabhattai* SP20 was detected when it was inoculated alone and not in the co-inoculations, plus in the EB3 treatment, where it was not added, although in low abundance. In contrast, *P. oryzihabitans* RL18 was not detected in any of the treatments. The most similar ASV ("faeda86d11b5aa509877ab3fc8a637cc") had a 94 % similarity percentage. A phylogenetic tree of all the ASVs classified in the taxonomic family Pseudomonadaceae confirmed that the ASVs belonged to different species of *Pseudomonas* other than *P. oryzihabitans* (SI Fig. S3). The datasets were analyzed to determine the number of ASVs assigned to *Pseudomonas*, *Bacillus Brevibacterium* (Fig. 4). The results indicated a statistically significant increase ($p < 0.0001$) in the number of ASVs classified as *Pseudomonas* in the treatment EB3 + RL18, while *Brevibacterium* was increased ($p < 0.0001$) in the ALL treatment. Additionally, sequences assigned to *Bacillus* were higher in the treatments EB3 ($p = 0.01$) and RL18 ($p < 0.0001$); however, *Bacillus* SP20 was not inoculated in those treatments.

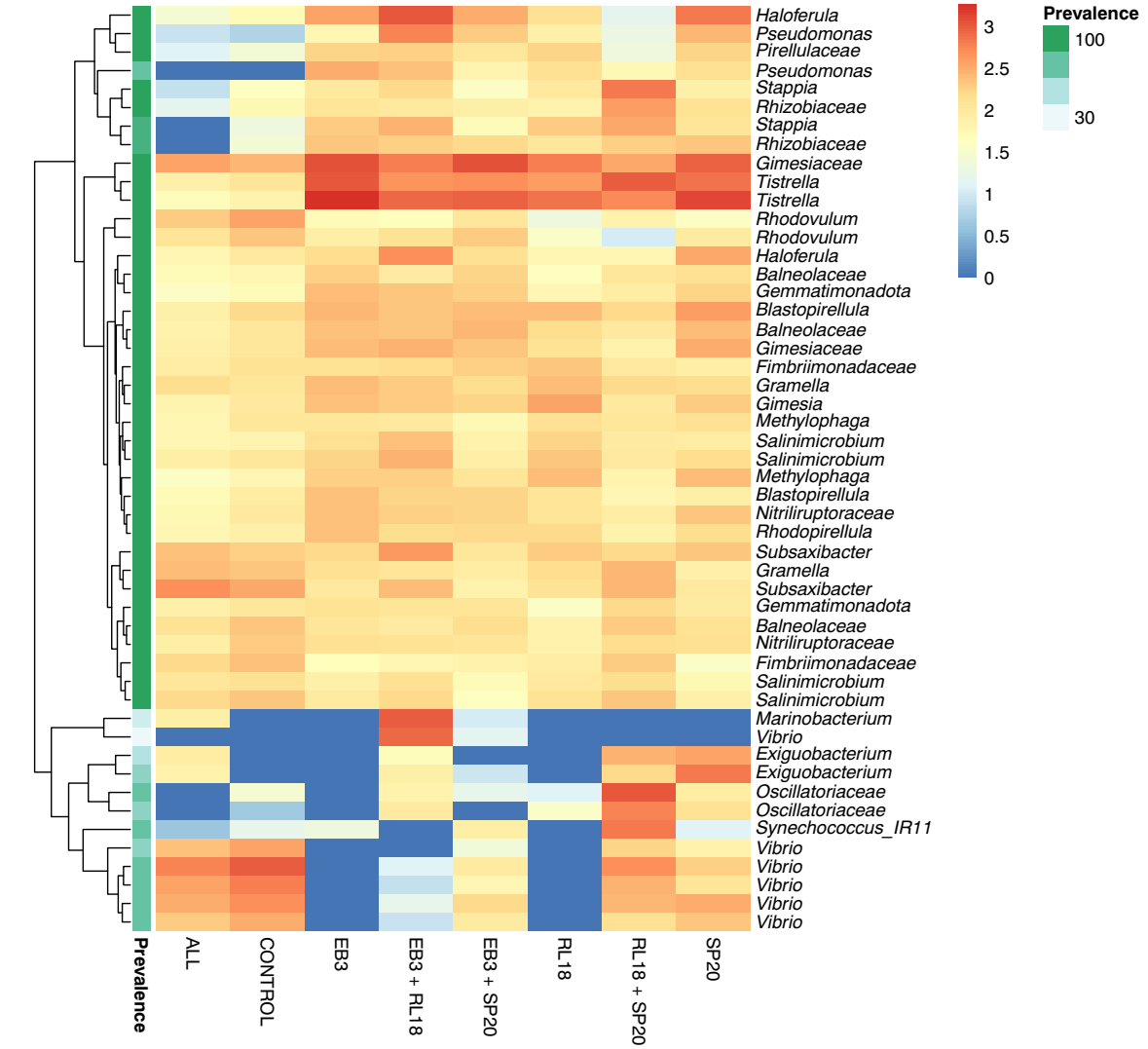

Fig. 3. Heatmap of the log abundance of the top 50 ASVs, classified at the highest possible taxonomic level, in the rhizosphere of *S. europaea* corresponding to different inoculation treatments. EB3: *Brevibacterium casei* EB3; RL18: *Pseudomonas oryzihabitans* RL18; SP20: *Bacillus aryabhattai* SP20; EB3 + RL18: *B. casei* EB3 and *P. oryzihabitans* RL18; EB3 + SP20: *B. casei EB3* and *B. aryabhattai* SP20; RL18 + SP20: *P. oryzihabitans* RL18 and *B. aryabhattai* SP20; ALL: *B. casei* EB3, *P. oryzihabitans* RL18 and *B. aryabhattai* SP20; CONTROL: non-inoculated.

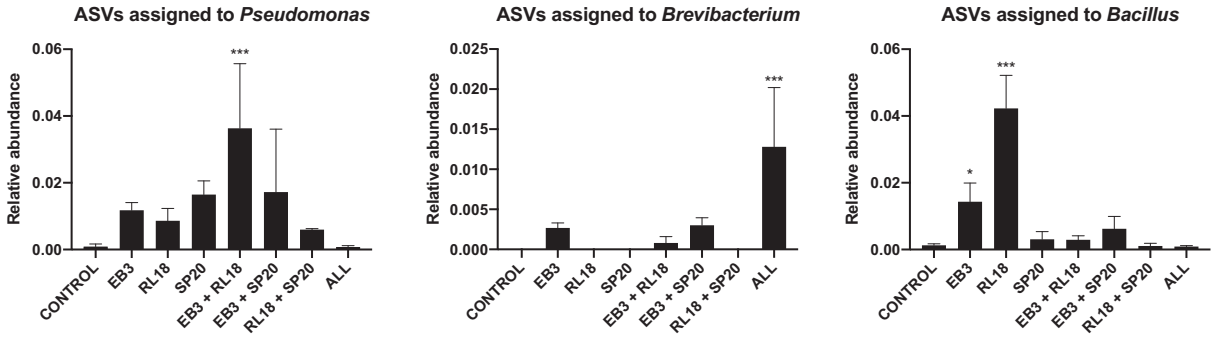

Fig. 4. Number of ASVs assigned to the taxonomic genus *Pseudomonas* sp., *Brevibacterium* sp. and *Bacillus* sp. detected in the datasets of the rhizosphere sequences of *S. europaea* in each inoculation treatment. Significance code: *** $p < 0.0001$, * $p < 0.01$.

3.4. Differentially abundant taxa across inoculation treatments

Fig. 5 shows the relative abundances of the differential ASVs identified in the three inoculation treatments that enhanced plant growth (EB3, EB3 + RL18 and ALL) compared to the non-inoculated control (Table 1). The pattern of enriched taxa in the treatments EB3 + RL18 (Fig. 5B) and ALL (Fig. 5C) was very similar. In both inoculation treatments, several ASVs identified as *Marinobacterium*, *Vibrio* and an ASV

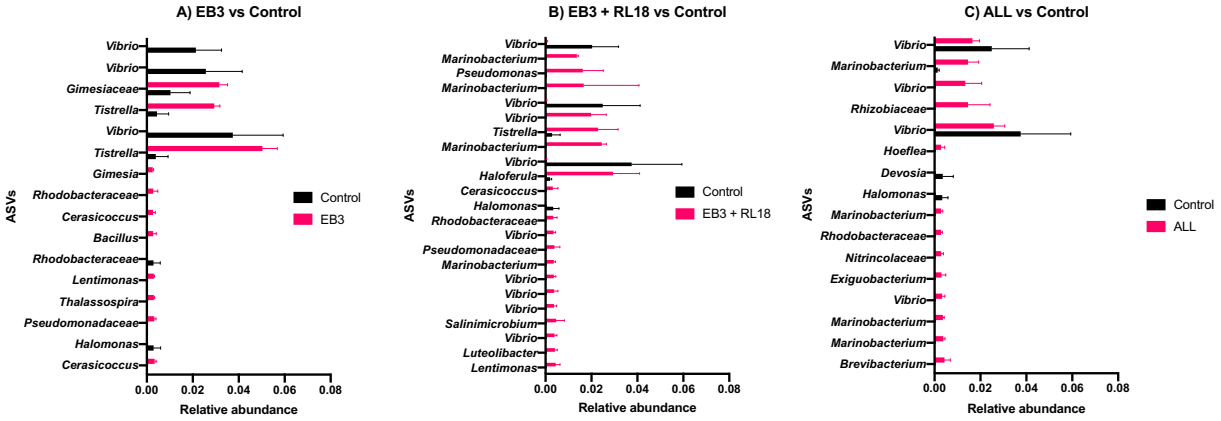

Fig. 5. Differential abundance of the ASVs that differ in abundance (P-adjusted <0.01) in each inoculation treatment, in relation to the non-inoculated control. EB3: *Brevibacterium casei* EB3; RL18: *Pseudomonas oryzihabitans* RL18; SP20: *Bacillus aryabhattai* SP20; EB3 + RL18: *B. casei* EB3 and *P. oryzihabitans* RL18; EB3 + SP20: *B. casei EB3* and *B. aryabhattai* SP20; RL18 + SP20: *P. oryzihabitans* RL18 and *B. aryabhattai* SP20; ALL: *B. casei* EB3, *P. oryzihabitans* RL18 and *B. aryabhattai* SP20; CONTROL: non-inoculated.

classified as Rhodobacteraceae were enriched in the rhizosphere of the inoculated plants compared to the non-inoculated control. Likewise, the co-inoculation EB3 + RL18 and the inoculation with EB3 included ASVs in common identified as *Tistrella*, *Lentimonas*, *Cerasicoccus*, Rhodobacteraceae and Pseudomonadaceae. However, the rhizosphere of plants inoculated with EB3 + RL18 was enriched exclusively with ASVs identified as *Pseudomonas*, *Salinimicrobium*, and *Luteolibacter*. Furthermore, ASVs classified as *Brevibacterium*, Nitrincolaceae, *Exiguobacterium* and *Hoeflea* were enriched exclusively in the ALL treatment. Lastly, the inoculation treatment of EB3 showed higher abundances of ASVs classified as Gimesiaceae, *Gimesia* and *Bacillus*. On the other hand, the non-inoculated control was enriched with ASVs classified as *Vibrio* and *Halomonas* in all comparisons with effective inoculation treatments.

3.5. Co-occurrence networks

EB3 was the only strain detected in the networks after the data filtering and running with sparcc cut-off 0.3. The low abundance of SP20 in treatments EB3 and SP20 (0.005 % and 0.01 %, respectively) reported in Ferreira et al. (2023) may have prevented the detection of this strain when constructing the co-occurrence networks. The summary of the network analysis is displayed in Table 2. Networks from bacterial communities inoculated with EB3 (EB3, EB3 + RL18, EB3 + SP20) had a higher edge-to-node ratio than the control and the other strain combinations, demonstrating that EB3 is a central hub in the plant microbial networks. For the ASV classified as the EB3 strain, we extracted the hub scores value in each network and found that the number of connections and centrality of the strain is higher in treatments with EB3+ RL18 (Table 2).

4. Discussion

Advances in DNA sequencing technologies allow a significant step forward in the detailed characterization of the complex cascade of effects of inoculants. In this work, we took advantage of powerful sequencing technologies and a factorial experimental design to investigate the impact of inoculations and co-inoculations with specific PGPB on the rhizosphere microbiome. While many studies have shown the benefits of bacterial inoculants on plant growth and yields, most have focused on using PGPB as biostimulants compared to fertilizers (Adesemoye et al., 2008; Assainar et al., 2018). However, few studies have evaluated the effects of PGPB on both plant growth and microbiomes (Ambrosini et al., 2016). Here, we investigated the effects of single and co-inoculations of PGPB on the rhizosphere microbiome of *S. europaea*. We found that the overall bacterial taxonomic composition at the class level was consistent with other descriptions of *S. europaea* root microbiome, with Alphaproteobacteria and Gammaproteobacteria representing the dominant members, followed by Bacteroidia (Yamamoto et al., 2018; Yuan et al., 2016). However, there were significant differences between the communities corresponding to different inoculation treatments, with inoculation explaining about 74 % of the total variability.

4.1. Overall effect of single inoculations and co-inoculations on bacterial communities in the rhizosphere

As measured by observed ASVs, the richness was significantly high in the treatment with the single inoculation of the bacterium *B. casei* EB3. The successful establishment of relationships between this bacterium, the plant and other bacterial members of the communities can explain this increase, supported by the effective colonization and survival of *B. casei* EB3 (Table 1) and the increase in the connections with other

Table 2
Summary network analysis.

Network	Nodes	Vertices	Ratio nodes/vertices	EB3 neighbors	EB3 Hub score
EB3 + RL18	1137	537,825	473.02	1006	0.943
ALL	724	217,247	300.06	616	0.924
EB3	1211	602,479	497.51	974	0.882
EB3 + SP20	1114	513,165	460.65	894	0.881
RL18 + SP20	892	328,139	367.87		
RL18	963	387,549	402.44		
SP20	1069	464,140	434.18		
CONTROL	856	297,276	347.29		

bacterial species (Table 2). Previous research has shown that *B. casei* strains can successfully colonize the rhizosphere soil of white mustard plants and alter the plant-associated microbial community (Płociniczak et al., 2016). Our study revealed that the structure of rhizobacterial communities, as assessed by 16S amplicon data, was significantly altered by single or co-inoculations with *B. casei* EB3 compared to control plants. These altered communities exhibited a general similarity pattern (treatments EB3, EB3 + RL18 and EB3 + SP20 in Fig. 1B), except when *B. aryabhattai* SP20 was also introduced in the inoculum (ALL treatment in Fig. 1B). In contrast, the inoculation of *Pseudomonas* RL18 resulted in different community structures depending on whether it was single inoculated or with EB3 or SP20 (Fig. 1B). The effects of inoculation with species of *Pseudomonas* sp. on bacterial diversity of the rhizosphere of plants are contradictory. For example, Jiménez et al. (2020) observed a decrease in overall bacterial diversity over time after a single inoculation with *Pseudomonas fluorescens* in different crops, such as rapeseed, corn gromwell and soybean. However, Ke et al. (2019) found no significant differences in diversity after inoculating maize with the diazotrophic *Pseudomonas stutzeri* and Roquigny et al. (2018) reported similar results after single inoculations of potato with *P. fluorescens*. In our study, the inoculation of the strain *P. oryzihabitans* RL18, even though it was not detected at the end of the experiment, altered the rhizobacterial communities by decreasing the richness, suggesting a substantial effect on bacterial interactions (e.g., competition, antagonism). In fact, among the single inoculations, RL18 was the second strain that most contributed to the variation in community structure (after EB3). On the other hand, *B. aryabhattai* SP20 had the least effect on structure when single inoculated but a distinct effect when co-inoculated with one or the other two strains, especially when combined with RL18. For example, the community structure of the inoculation treatments EB3, SP20, or the combination of both was very similar, but completely different rhizobacterial communities resulted when SP20 was co-inoculated with RL18.

The interaction between *Pseudomonas* and *Bacillus* strains is context-dependent, influenced by nutrient sources, temperature, isolation source, phylogeny, and secondary metabolites (Lyng and Kovács, 2023). Some interactions are positive, neutral, or negative (Lyng and Kovács, 2023). When isolated from plants, *Pseudomonas* is often more competitive than *Bacillus*, but antagonistic interactions from *Bacillus* to *Pseudomonas* have also been reported (Boopathi et al., 2022; Garbeva et al., 2011). In our experiments, *P. oryzihabitans* RL18 and *B. aryabhattai* SP20 did not show antagonistic activity in vitro (Ferreira et al., 2023). However, when co-inoculated (treatments RL18 + SP20 and All), neither *P. oryzihabitans* RL18 or *B. aryabhattai* SP20 were detected in the rhizosphere (Table 1). Furthermore, neither *Bacillus* nor *Pseudomonas* was significantly abundant in those treatments (Fig. 4). This suggests that *B. aryabhattai* SP20 and *P. oryzihabitans* RL18 may have interacted negatively with each other. Interestingly, the combination of both strains had the most significant effect on the variability of the rhizosphere microbiome (20 %; Supplementary Table S6). The main difference in this treatment was the dominance of Acidobacteriae and Cyanobacteriia, which are common beneficial members of the rhizosphere and endosphere of halophytic plants (Kielak et al., 2016; Mishra et al., 2021; Tian and Zhang, 2017; Yamamoto et al., 2018). However, this treatment failed to induce a significant plant growth enhancement, suggesting that other factors may have interfered with the activity of these bacteria.

The comparative analysis of the 16S sequencing data revealed remarkably similarities between the rhizosphere bacterial communities of ALL treatment and CONTROL plants. This similarity is evident in both the overall community structure, as demonstrated by the PCoA plot (Fig. 2B) and the specific composition, as portrayed by the heatmap (Fig. 3). This aligns with the minimal impact of the co-inoculation of all PGBP strains on community composition, with only a 6 % change observed (Supplementary table S6). The generalized use of PGPB as biostimulants raises the concern of undesirable changes in soil microbial community structure and functional diversity. Ideally, biostimulants should enhance plant productivity with minimal disruption of the indigenous microbiome. Our findings indicate that the inoculation of the three PGPB strains may have the desired effects on plant growth without significantly altering the rhizosphere in *S. europaea*.

4.2. Rhizobacterial composition in plants with enhanced growth

In terms of biomass production, the most successful treatment corresponded to the co-inoculation with *B. casei* and *P. oryzihabitans* (EB3 + RL18), followed by the co-inoculation with the three strains (ALL), and the single inoculation with *B. casei* (EB3). *B. casei* is the common inoculated element, and since it was detected in the rhizosphere, it may suggest a direct role in regulating plant growth. Aside from the PGP traits that characterized this strain (nitrogen fixation, production of ACC deaminase and IAA), our results also evidenced that the inoculation of *B. casei* EB3 increases rhizosphere richness and network complexity, indicating *B. casei* EB3 functions as a microbial hub that recruits beneficial microorganisms (Agler et al., 2016). Contrastingly, ASVs matching *P. oryzihabitans* RL18, one of the partners in the most successful treatments (EB3 + RL18 and ALL), were not detected in any of the treatments. Inoculation with this strain, however, was associated with an enrichment in other Pseudomonads. Higher densities of *Pseudomonas* strains in the rhizosphere of tomato plants have shown beneficial effects on plant growth in tomato plants (Hu et al., 2021). Therefore, the observed improvement in the growth of *S. europaea* with the EB3 + RL18 and ALL treatments may be due to the synergic combination of direct effects of *B. casei* EB3 and indirect effects of *P. oryzihabitans* RL18, mediated by biotic relations, that will lead to the selective enrichment of other members of the community including Pseudomonads.

The analysis of 16S gene sequences revealed that the bacterial taxa that exhibited significantly higher abundances in the treatment that most enhanced plant growth (EB3 + RL18) belonged to *Marinobacterium*, *Vibrio*, and *Pseudomonas* (Figs. 3–5). Some of these bacteria have been isolated from saline-associated plants and some have been shown to promote plant growth and alleviate stress in other halophytic or non-halophytic plants. For example, *Vibrio* and *Pseudomonas* sp. isolated from *Salicornia brachiata* have shown to alleviate salt stress and stimulate growth (Jha et al., 2012), the inoculation with *Vibrio spartinae* can improve the growth of another halophyte, *Halimione portulacoides,* under saline conditions (Mateos-Naranjo et al., 2020). The *Marinobacterium* genus has been identified in the rhizosphere of *Sueda japonica* (Kim et al., 2008), and the *Marinobacterium* sp. isolated from the halophyte *Psoralea corylifolia* L. has been shown to increase wheat growth and salinity tolerance (Sorty et al., 2016). These findings suggest that the inoculation with *B. casei* EB3 and *P. oryzihabitans* RL18 may have altered the rhizosphere by favoring the development of similar beneficial bacterial populations of *Pseudomonas*, *Vibrio* and *Marinobacter* that potentially promote growth and mitigate salinity in *S. europaea*.

Likewise, the ALL treatment, which also significantly enhanced plant growth compared to control, showed higher abundances of the same ASVs in EB3 + RL18 treatment identified as *Marinobacterium* sp., *Exiguobacterium* sp. and *Vibrio* (Figs. 3, 5) as well as *Brevibacterium* (Figs. 4–5).

In order to investigate whether the strain *B. casei* EB3 has effectively increased the number of connections (nodes/vertices) with these genera, we mapped the ASVs classified as *Marinobacterium*, *Vibrio*, *Bacillus*, *Brevibacterium* and *Pseudomonas* on the networks Fig. 6 shows that EB3 was highly connected to *Marinobacterium*, *Vibrio*, and *Pseudomonas* in the EB3 + RL18 treatment, while in the ALL treatment, EB3 was more highly connected to *Marinobacterium* and *Vibrio*. Single inoculation with EB3 resulted in higher connectivity with *Bacillus*, *Marinobacterium*, and *Pseudomonas* but less with *Vibrio*.

Interestingly, the highest hub scores (Table 2) were for plants of the most significant enhanced growth (EB3 + RL18 and ALL). PGP beneficial traits in EB3 and RL18, such as the production of ACC-deaminase,

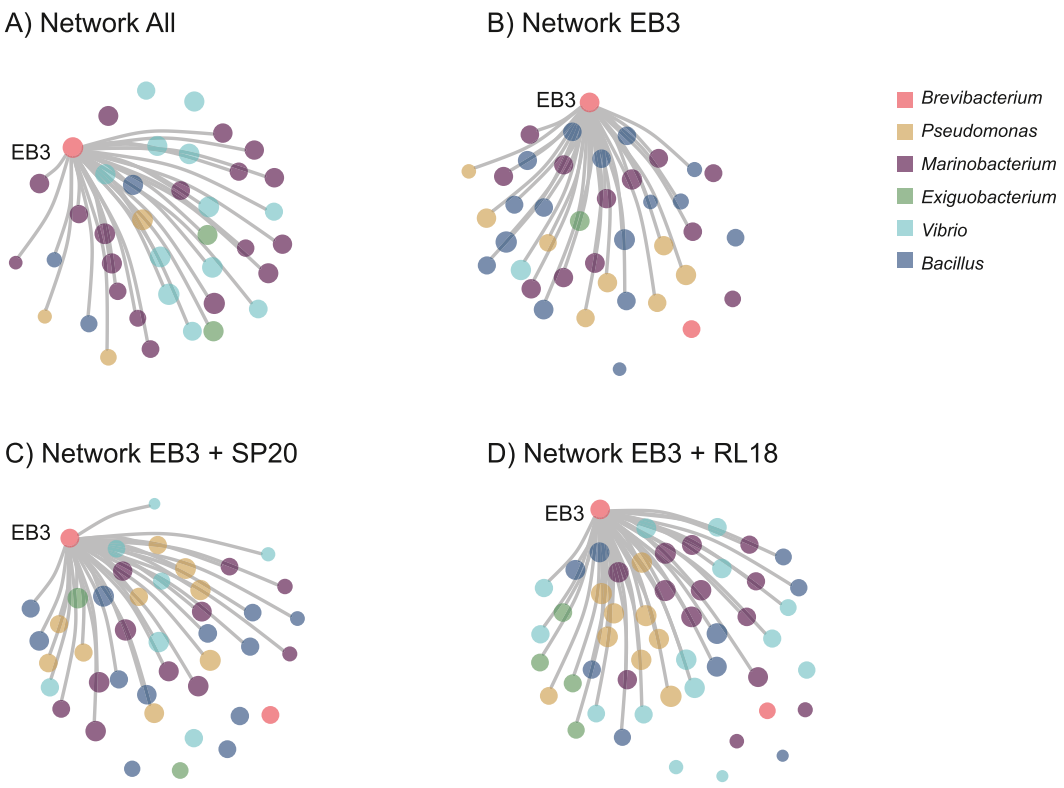

Fig. 6. Induced subgraphs from networks with the strain *B. casei* EB3. Induced subgraphs generated using the Large Graph Layout generator in igraph. Each node represents a bacterial ASV where the size is proportional to the hub scores within the induced network. Nodes of interest are color-coded by genus. Only edges connected to EB3 are shown for clarity. Links between the nodes show statistically significant positive Spearman correlations ($p < 0.05$).

can be horizontally transferred between bacteria (Nascimento et al., 2014), and in addition, the sub-products of the conversion of ACC into α-ketobutyrate and ammonia can also be used by other associated microorganisms as substrates for growth (Nascimento et al., 2018). Therefore, establishing symbiotic or synergistic mutually beneficial relationships with the inoculated PGPB might be a survival strategy for some microbes, contributing to the plant growth-promoting effects (Kong and Liu, 2022). In this study, the high number of connected nodes in the networks suggests a more stable community, which may have led to the enhanced growth of *S. europaea* biomass. For the other strains that were not detected in the rhizosphere at the end of the experiment, we believe that they may have been outcompeted or inhibited by other microbial members of the community.

In this study, we investigated the effects of different PGPB inoculations and co-inoculations on the rhizosphere microbial communities and the growth of *S. europaea* plants. Our results based on barcoding analyses showed that all the inoculations and co-inoculations modified the rhizosphere microbial communities of *S. europaea* to different degrees. The consortia containing all the strains had the least disruptive effect on the indigenous microbiome compared to single or dual-strain inoculants. The *B. casei* EB3 strain was the most effective plant growth-promoting bacterium, evidenced by its ability to colonize the rhizosphere and form a complex network of interactions with other members of the community, either when inoculated in single or in co-inoculations with other strains. In the inoculations that enhanced plant growth (co-inoculation of EB3 + RL18 and ALL), common bacterial members were found in the rhizosphere of these plants, including *Marinobacterium*, *Vibrio* and *Pseudomonas* sps. These bacterial populations can reduce saline stress while contributing to nutrient supply. However, the enhanced plant growth was explained by the direct beneficial traits from *B. casei* EB3 and its synergistic associations with other beneficial bacteria. As pointed out by other authors (Compant et al., 2019), the results highlight the need for a smart and knowledge-driven selection of consortia and strains to develop consistent formulations and predictable biostimulation outcomes. Our findings suggest that PGPB consortia can promote plant growth by modifying the rhizosphere microbiome and forming synergistic associations with other beneficial bacteria.

CRediT authorship contribution statement

Isabel N. Sierra-García: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Maria J. Ferreira:** Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. **Adriana Torres-Ballesteros:** Formal analysis, Software, Writing – review & editing, Visualization. **Antonio Louvado:** Methodology, Supervision, Writing – review & editing. **Newton Gomes:** Conceptualization, Project administration, Resources, Supervision, Writing – review & editing. **Angela Cunha:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Sequences used in this study have been uploaded to the NCBI Short Read Archive under the BioProject number: PRJNA858439.

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Appendix A. Supplementary data

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