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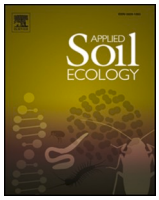

Research paper

Unveiling changes in the rhizosphere bacteriome of sunflower (*Helianthus annuus* L.) inbred lines linked to their resistance to the soil borne pathogen *Verticillium dahliae* Kleb

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ABSTRACT

Plants infected by fungal pathogens often actively recruit microbes in their roots to protect themselves. It is widely accepted that the plant microbiome plays a crucial role to sustain the fitness, resilience and development of the plant holobiont, and that the host actively shapes the rhizosphere microbiome to prevent or suppress soil borne diseases. Here, we studied the rhizosphere bacterial communities of three sunflower inbred lines (ILs) with different level of resistance to *Verticillium dahliae* Kleb. We used *16S rRNA* gene amplicon sequencing to profile bacterial communities before and after inoculation with *V. dahliae*. Overall, there was no significant association of alpha diversity indices with sunflower ILs and inoculation of the pathogen. However, a moderate increase in the richness and diversity was observed with the increment of resistance. In contrast, there were clear differences in the rhizosphere bacterial community structures related to the level of susceptibility of the sunflower genotypes. The predominant phyla comprised Proteobacteria, Bacteroidetes, and Acidobacteria. At the genus level, *Rhodanobacter*, *Chujaibacter*, *Flavitalea*, *Lysobacter*, *Devosia*, *Bryobacter*, *Dokdonella* and *Bradyrhizobium* were the most abundant genera in all cases. Our results indicate that sunflower genotype and *V. dahliae* infection of roots led to considerable changes in the composition of the rhizosphere bacterial communities. Changes were mostly observed in the relative abundances of core microbiome members, which varied significantly and differentially depending on the sunflower ILs and its level of resistance to *V. dahliae*. Thus, harnessing sunflower-associated rhizosphere bacteriomes for disease control may offer a valuable alternative strategy to increase the productivity and sustainability of agricultural production for this crop.

1. Introduction

Sunflower Verticillium wilt and leaf mottle (SVW), caused by the fungus *Verticillium dahliae* Kleb. is one of the most extensive disease of sunflower in Argentina, Canada and the United States (Gulya, 2007; Harveson et al., 2016; Montecchia et al., 2021; Pereyra and Escande, 1994; Radi and Gulya, 2007; Sackston, 2009). Furthermore, it is becoming a major constraint to sunflower production in temperate European countries due to increasing incidence in France, Italy, Spain, and countries around the Black Sea (Bret-Mestries et al., 2021; Martín-Sanz et al., 2018). *V. dahliae* is a soil-borne and seed-borne fungal pathogen affecting over 300 species of dicotyledonous plants including many agriculturally important crops and woody plants (Fradin and Thomma, 2006; Inderbitzin et al., 2011). The management is difficult through common agricultural practices. Fungal inoculum consists of long-lasting microsclerotia, which remain viable in soil from 10 to 15 years (Fradin and Thomma, 2006; Inderbitzin and Subbarao, 2014; Klosterman et al., 2009; Pegg and Brady, 2002).

In Argentina, *V. dahliae* is an endemic pathogen with four characterized races on sunflowers (Clemente et al., 2017; Galella et al., 2004). The fungus is disseminated over 1.2 million hectares, affecting over 70 % of the country's sunflower growing region (Montecchia et al., 2021). Despite the prevalence of the pathogen, the disease-management tools available to date are limited. The implementation of an integrated

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disease management, such as, the combination of no-tilling and the use of resistant cultivars is still the most effective strategy to control SVW (Montes-Osuna and Mercado-Blanco, 2020; Quiroz et al., 2008). Currently, disease management in sunflower is effectively achieved through a selective breeding program aimed at developing heritable resistance to the pathogen (Domínguez, 2022; Filippi et al., 2014, 2022; Montecchia et al., 2021; Talukder et al., 2014).

The rhizosphere is defined as the narrow zone of soil surrounding the plant root, which hosts microbial populations distinct from those in the surrounding bulk soil. The main factor to differentiate the rhizosphere from bulk soils is due to plant root rhizodeposition making the rhizosphere a nutrient-richer ecological niche than the bulk soil (Ling et al., 2022; Park et al., 2023). The rhizosphere microbiome may provide a range of beneficial functions to the plant host including nutrient acquisition, abiotic stress tolerance, and protection against soil-borne pathogens (Bulgarelli et al., 2013; Chepsergon and Moleleki, 2023; Lei et al., 2019; Mendes et al., 2018).

The assembly and function of the rhizobacterial community are determined by several factors, such as plant species, cultivar, plant developmental stage, and soil properties (Gao et al., 2021; Park et al., 2023; Pascale et al., 2020; Trivedi et al., 2020).While different plant species, or even distinct genotypes of the same species, tend to recruit relatively distinct rhizobacterial communities (Berg et al., 2001; Bulgarelli et al., 2013; Park et al., 2023), these communities often share a core microbiome even in different environments across geographical regions (Bouffaud et al., 2014; Matthews et al., 2019; Trivedi et al., 2020). Several studies have shown that plant genotypes with differential resistance or tolerance to a given pathogen may boost the abundance of particular microbial taxa in the rhizosphere, thereby influencing host performance against that pathogen (Cardoni et al., 2023; Mendes et al., 2018; Pogoda et al., 2023; Wei et al., 2021).

One of the most addressed benefits of plant growth-promoting microorganisms found in different soils is the suppression of soil-borne pathogens (Bai et al., 2015; Finkel et al., 2017; Mendes et al., 2011; Wei et al., 2021; Xiong et al., 2017). However, while there have been some studies exploring the sunflower rhizosphere microbiome (Balogun et al., 2023; Nwachukwu et al., 2023; Nwachukwu et al., 2022; Pogoda et al., 2023), none have addressed the impact of SVW on the rhizosphere. In this context, accurate knowledge of the structure and composition of the rhizosphere bacteriome associated with sunflower germplasm showing differential responses to SVW is essential to propose future studies that will elucidate to what extent these bacterial communities contribute to resistance/susceptibility to *V. dahliae*. Functional studies on plant-microbiome interactions can be proposed based on the information provided by current state of the art. The knowledge gained will provide a solid basis for the development of innovative strategies, such as microbiome breeding, that integrate microbiomes into plant breeding programs and sustainable crop protection strategies (Mueller et al., 2021; Mueller and Linksvayer, 2022).

In this study, we evaluated the diversity, structure and taxonomical profile of the rhizosphere bacteriome of three sunflower inbred lines associated with the occurrence of Verticillium wilt. These inbred lines were previously shown to have high, moderate, and low susceptibility to *V. dahliae* (Montecchia et al., 2021). We hypothesized that (1) sunflower inbred lines with different level of resistance to SVW recruit distinct bacterial communities, and (2) the introduction of *V. dahliae* alters the rhizosphere bacteriome that may contribute to the cultivar response to SVW. To test this, we conducted comparative bacteriome analyses that revealed distinct patterns in the structure of the bacterial communities inhabiting the rhizosphere of the three sunflower inbred lines.

2. Materials and methods

2.1. Sunflower plant material

Three sunflower inbred lines (ILs) with differing resistance level against SVW were used in this study: two public lines, RHA266 and RHA439, and an IL from INTA's (Instituto Nacional de Tecnología Agropecuaria) Association Mapping Population (AMP) PMA26. These lines were previously characterized as susceptible (S), moderate (M), and highly resistant (R) to Argentinian *V. dahliae* races, respectively (Montecchia, 2019; Montecchia et al., 2021). The ILs examined in this study are deposited in the Active Germplasm Bank of EEA-INTA Manfredi.

2.2. Greenhouse experiment design

Two-week-old sunflower seedlings grown in perlite, from each ILs, were inoculated by immersing the roots in a conidial suspension (1×10^6 conidia per mL) of Argentinian *V. dahliae* race 1, strain Colón (Galella et al., 2004) for 16 h in darkness at room temperature. At the same time, the control plants were mock-inoculated using sterile water. Afterward, the plants were transplanted into 5 L plastic pots with peat-based substrate (GrowMix® *Z*- Floricultura Específico, Terrafertil, Buenos Aires, Argentina), to avoid the effects of chemical compounds such as fertilizers, pesticides, insecticides from agricultural field management (Wolfgang et al., 2020), and grown in greenhouse benches at 25 ± 2 °C under a 14 h photoperiod. During this time, plants were watered as needed with tap water. A randomized complete block design with nine replicates was selected for the experiment. Each block consisted of fourteen replicated pots (one plant per pot) for inoculated and non-inoculated treatment of each plant genotype, respectively. For bulk soil analysis, fourteen pots without plants were included in each block. Three replicates of bulk soil and rhizosphere samples were collected from randomized blocks at 30 days post-inoculation (dpi).

Given that SVW symptoms appear at least 20 days post inoculation (dpi) in the susceptible genotype, 5 plants per block and treatment were maintained for two months in the greenhouse to evaluate disease progress and confirm the deleterious effect caused by the pathogen.

2.3. Rhizosphere and bulk soil samples collection

At 30 dpi, one plant from each block per treatment and genotype was randomly selected and carefully uprooted. Root system of the sunflower plants was first vigorously shaken to remove loosely adhering soil particles.

The rhizosphere collection was performed according to the protocol described by Simmons et al. (2018) with some modifications. Briefly, root samples were placed in 50 mL centrifuge tubes. Each tube was filled up to 30 mL with epiphyte removal buffer (0.75 % KH_2PO_4, 0.95 % K_2HPO_4, 1 % Triton X-100 in ddH_2O) and then shaken at 270 rpm for 30 min (room temperature). The rhizosphere fraction was collected by centrifuging the root wash mixture at 4000 rpm at 4 °C for 20 min. The supernatant was discarded, the pellets were immediately frozen with liquid nitrogen and lyophilized before DNA extraction.

The bulk soil sample from each pot (without plants) was taken at 5 cm depth from the soil surface. Then three rhizosphere and bulk soil samples, from the same treatment and plant genotype, were combined as a single composite sample resulting in three biological replicates.

2.4. DNA isolation and next-generation sequencing

DNA extraction from rhizosphere and bulk soil samples (250 mg each) was performed using the MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's recommendations. DNA quality was analyzed by electrophoresis on a 0.8 % (*w/v*) agarose gel and quantified using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Pleasanton, CA, USA). DNA was stored at −80 °C until further processing.

The V5-V7 region of the *16S rRNA* gene was amplified and subsequently sequenced using bacterial universal primers 799F (5′ -AACMGGATTAGATACCCKG- 3′) and 1223R (3′ -CCATTGTAGTACGTGTGTA- 3′)

(Mauger et al., 2021). The primers were designed with Illumina overhang adapters. DNA amplicon libraries were first sequenced using the Illumina MiSeq instrument (Illumina Inc., San Diego, CA, USA) operating with v2 chemistry to obtain 2 × 250 bp paired-end reads at the Unidad de Genómica (UGB) of INTA (Hurlingham, Buenos Aires, Argentina). In addition, a second sequencing run with 2 × 300 bp paired-end protocol was performed on the same libraries with an Illumina MiSeq instrument (Illumina Inc., San Diego, CA, USA) at Macrogen, Inc. (Seoul, Republic of Korea).

2.5. Processing of sequencing reads

Demultiplexed files containing raw reads were processed using the DADA2 package (Callahan et al., 2016) of R Studio software, version 4.4.1 (R Core Team, 2021). The reads processing followed the micro4all package tutorial (Wentzien et al., 2023). The seqtabs generated for each sequencing run were combined using the function mergeSequenceTables prior to the chimera removal. The taxonomic classification of Amplicon Sequence Variants (ASVs) was achieved using the assignTaxonomy command against a modified version of the Ribosomal Database Project II, training set v.18 (Cole et al., 2014). Chloroplast, mitochondria and unknown at Kingdom level sequences were removed from ASV tables before statistical analyses. Finally, ASVs representing <0.005 % of the total reads were filtered out according to Bokulich et al. (2013).

2.6. Statistical analysis of sequencing data

All analyses were performed according to the micro4all tutorial (Wentzien et al., 2023). Alpha diversity was calculated based on Observed Richness, Shannon, Inverse of Simpson, and Pielou's Evenness indices. To avoid the potential biases in estimation of diversity, related to different sequencing depths among samples, a rarefaction step was performed. We analyzed the homogeneity of variances and normal distribution of alpha indices, by means of Levene's test and Shapiro-Wilk test. The statistical differences among groups of samples in the alpha indices were analyzed by performing two-way balanced ANOVA followed by a Tukey's HSD *post-hoc* test to perform multiple comparisons. Beta diversity was assessed based on Bray-Curtis and Unweighted Unifrac distances. Permutational Multivariate Analysis of Variance (PERMANOVA) and Multivariate Homogeneity of Groups Dispersions (BETADISPER) were performed. To visualize the results of PERMANOVA, the distribution of each group of samples in the multivariate space was plotted by Principal Coordinate Analysis (PCoA). A confidence level > 95 % was selected and applied for all the statistical analyses.

Further analysis was carried out to identify specific bacterial taxa that differed significantly among groups of samples using Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) (Lin and Peddada, 2020). The ancomloop function implemented in micro4all was executed in R. Those genera with a relative abundance <0.1 % per sample were excluded from the analysis. *P*-values of the comparisons were adjusted by implementing Holm's method included in the function.

The core bacterial taxa were defined as ASVs taxonomically classified at order level and present in at least in 67 % of the replicates of the entire dataset. We applied this approach using three different datasets, as follows: (1) we determined the core bacteriome for each treatment by identifying the ASVs shared among the three genotypes. This approach was applied to both the non-inoculated plants and to *V. dahliae*-inoculated plants, hence, we define the core bacteriome of non-inoculated and inoculates plants, respectively, (2) we determined the core of the entire dataset defined as the "core bacteriome of the sunflower rhizosphere" by comparing the ASVs present in the non-inoculated and inoculated shared cores defined previously, and (3) in addition to the core members, we described the "accessory bacteriome", to identify bacterial taxa associated with specific genotypes and treatments. After construction, core microbiomes were plotted in Venn diagrams with the function 'venn.diagram' of the package 'VennDiagram' (Chen and Boutros, 2011) in R.

3. Results

3.1. General characteristics of sequencing datasets

A total of 2.143.799 raw reads were generated across bulk soil and rhizosphere soil samples. After quality filtering, 553.142 sequences were clustered into 634 bacterial ASVs that were used for further analyses (Table S1). >58 % of the bacterial sequences were classified into 108 genera.

All the rarefaction curves calculated at the ASV level clearly reached to the asymptote, indicating that the sampling effort was enough and the diversity of bulk soil and rhizosphere bacterial communities will surely be covered (Fig. S1).

3.2. Alpha diversity

To investigate how bacterial alpha diversity was affected by sunflower genotype and the presence of *V. dahliae*, Observed Richness, Inverse of Simpson's index, and Shannon's Diversity index and Pielou's Evenness index were calculated for each rhizosphere sample and the bulk soil. We observed a pattern of increased alpha diversity indices along the resistance level of the three genotypes, and among treatments (Fig. 1). Diversity of bacterial communities, determined using the Inverse Simpson's index, was significantly higher in the bulk soil (BS) than in the rhizosphere from S-NI and S—I (balanced ANOVA, Tukey's *post-hoc* test, $P = 0.04$). In addition, the Evenness index was statistically significantly higher in BS than in the rhizosphere of S—I, M-I and R-I (balanced ANOVA, Tukey's *post-hoc* test, **$P = 0.003$, $P = 0.007$ and $P = 0.033$, respectively;** Fig. 1).

3.3. Beta diversity

Differences in community structure (ASV level) between the rhizosphere of non-inoculated plants and bulk soils were observed via the Principal Coordinates Analysis (PCoA) on unweighted UniFrac distance. These differences were further supported by PERMANOVA ($R^2 = 0.50$, *P*

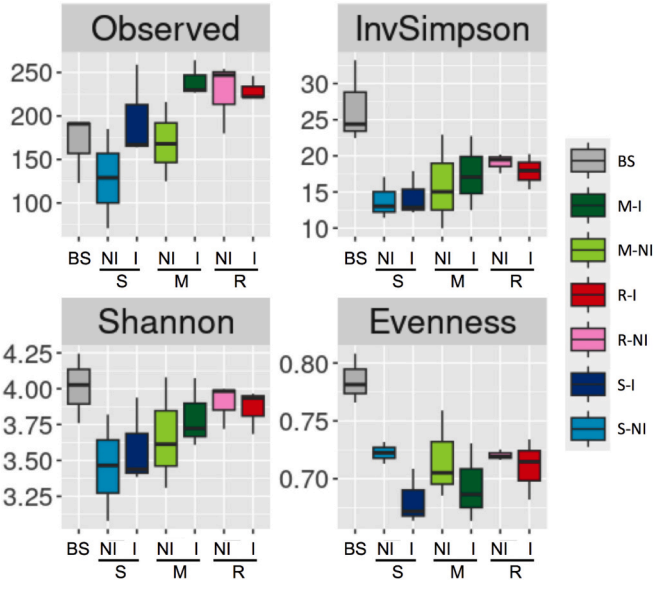

Fig. 1. Alpha-diversity indices of bulk soil and rhizosphere soil samples associated with different sunflower genotypes non-inoculated (NI) and *Verticillium dahliae* inoculated (I). BS: bulk soil; S: susceptible; M: moderate; R: resistant.

= 0.002) (Fig. S2).

We found that rhizosphere samples primarily clustered by sunflower genotype, with the variation in beta diversity being explained to a greater extent by genotype (PERMANOVA, $R^2 = 0.40$, $P = 0.045$; $R^2 = 0.33$ $P = 0.014$, for non-inoculated and inoculated rhizosphere, respectively). No statistically significant differences were found between treatments (i.e. non-inoculated vs. *V. dahliae* inoculated) on the same inbred lines (Table S2). PCoA based on UniFrac distances revealed that the samples of non-inoculated rhizosphere bacteriome, from the three sunflower genotypes, were grouped together (Fig. 2A), which are separated along the first and second coordinate axes. After the inoculation with *V. dahliae*, the rhizosphere bacterial community composition from the M genotype changed along the left side of PCo1 (getting closer to R genotype microbiota) and the S genotype shifted on PCo2 (Fig. 2B).

3.4. Rhizosphere and bulk soil bacterial community composition

Proteobacteria, Bacteroidetes, and Acidobacteria dominated the taxonomic profile (phylum level) of the bacterial communities present in both the rhizosphere of the three examined sunflower genotypes and the bulk soil (Fig. 3A, Table S3). These phyla accounted for at least 98 % of all sequences. For instance, statistically significant differences (ANCOM-BC test, $P < 0.05$) were observed for Bacteroidetes between non-inoculated S and M, and Proteobacteria in S non-inoculated rhizospheres concerning the BS. In addition, statistically significant differences were found in the abundance of Proteobacteria between the non-inoculated R compared to the *V. dahliae*-inoculated rhizosphere (ANCOM-BC test, $P = 10^{-8}$). No archaeal sequences were detected in the whole dataset.

At the genus level, bacterial communities were mainly represented by members of the genera *Rhodanobacter*, *Chujaibacter*, *Flavitalea*, *Lysobacter*, *Devosia*, *Bryobacter*, *Dokdonella*, and *Bradyrhizobium* (Fig. 3B, Table S3). Among these, *Rhodanobacter*, *Chujaibacter*, and *Flavitalea* accounted for >30 % of the sequences in all treatments and genotypes.

3.5. Core bacteriome identification

Venn diagrams were employed to illustrate the patterns of ASVs within the rhizosphere bacteriomes observed across sunflower genotypes and treatments. We found that only 62 out of 456 ASVs (13.60 %) were shared among the three genotypes in the non-inoculated rhizosphere. The ASVs that made-up the non-inoculated core primarily belonged to the order Xanthomonadales (32.30 %), Rhizobiales (30.7 %), Chitinophagales (11.3 %), Sphingomonadales (8.1 %), and order *incertae sedis* belonging to the Acidobacteria class Gp3 (4.8 %) (Fig. 4A, Table S4). In contrast, the rhizosphere communities of *V. dahliae*-inoculated plants shared a higher number of ASVs: 119 out of 419 total ASVs (28.40 %). This latter core is primarily composed by the order Rhizobiales (31.1 %), Xanthomonadales (15.13 %), Chitinophagales (13.45 %), and Sphingomonadales (9.24 %) (Fig. 4B, Table S4). Furthermore, we observed a significant number of ASVs seemed to be unique of each genotype. The composition of the accessory core changed under the presence of *V. dahliae,* among which the most abundant genera belonged to the orders Rhizobiales, Xanthomonadales, Chitinophagales, and Sphingomonadales (Table S4).

We extended our analysis to determine which ASVs are stable across rhizosphere microbial communities of the non-inoculated and *V. dahliae*-inoculated cores. The resulting core bacteriome is made up of 56 ASVs of which 88.71 % and 46.22 % belonged to non-inoculated and inoculated plants, respectively. The bacterial taxa of sunflower rhizosphere core bacteriome primarily belonged to the families Rhodanobacteraceae (28.60 %), Chitinophagaceae (12.50 %), Braddyrhizobiaceae (10.71 %), family *incertae sedis* belonging to Acidobacteria class Gp3, and Xanthomonadaceae (7.14 %) (Fig. 4C, Table S4).

3.6. Genotype-dependent selection of rhizosphere bacteria

The ANCOM-BC resulting analysis of the non-inoculated rhizosphere revealed 20 genera that exhibited greater abundance in the M and R genotypes relative to the bulk soil (ANCOM-BC, $P < 0.05$). Furthermore, bacterial communities of these genotypes displayed a similar taxonomic pattern. This analysis also revealed four genera that exhibited greater abundances in the non-inoculated S rhizosphere than in the bulk soil (ANCOM-BC, $P < 0.05$). None of the four genera was found on the M and R genotypes (Fig. 5A, Table S5). In addition, we compared communities of the *V. dahliae*-inoculated rhizospheres to that of the bulk soil. Interestingly, the ANCOM-BC analysis showed several changes in the bacterial community in the rhizospheres from the three different genotypes. In the presence of *V. dahliae* the S rhizosphere displayed a microbial community more diverse and similar to the one of the M and R genotypes (Fig. 5B, Table S5).

When comparing between the rhizosphere of S-NI and S—I, nine genera exhibited significantly different abundances such as *Edaphobaculum*, *Variibacter*, *Ferruginibacter*, *Thermomonas*, *Rhizomicrobium*, *Pseudolabrys*, *Phenylobacterium*, *Micropepsis* and *Altererythrobacter* (Fig. 5C, Table S5). In the case of M genotype, only two genera, *Flavobacterium* and *Flavisolibacter*, showed significantly different abundances when *V. dahliae* was present (Fig. 5D, Table S5). Finally, regarding the comparison between R-NI and R-I rhizosphere we observed a significant increase in the abundance of *Dyella* and *Lysobacter* when plants were inoculated (Fig. 5E, Table S5).

Genera whose proportion in non-inoculated vs. inoculated rhizosphere was diminished belonged to a few orders such as Sphingomonadales and Rhizobiales in the S and M genotypes (ANCOM-BC, $P < 0.009$). In addition, Acidobacteria_Gp3_ois, Caulobacterales, and Micropepsales decreased in the S genotype (ANCOM-BC, $P < 0.01$). Meanwhile, Xanthomonadales showed a marginal difference in the R genotype (ANCOM-BC, $P = 0.053$).

4. Discussion

In this work, bacterial communities in the sunflower rhizosphere were studied by *16S rRNA* amplicon sequencing, to unveil their response to *V. dahliae* invasion and explore potential connections between disease resistance and bacteriome composition. To this end, we compared the bacteriome diversity and structure of three sunflower inbred lines with different levels of resistance (RHA266, susceptible; RHA439, moderate; and PMA26, resistant) to SVW.

Our results showed differences in bacterial alpha diversity associated with sunflower genotype and level of susceptibility to SVW. A decrease in diversity from the bulk soil to the rhizosphere was observed, suggesting that the genotypes analyzed here exert a selective influence on the belowground microbial community. This selective effect can be attributed, among other factors, to root system exudates that attract and thereby help to recruit specific soil-borne microorganisms (Xiong et al., 2021). This rhizospheric effect has been observed in different plant species such as corn (Brisson et al., 2019), tomato (Lee et al., 2019), bean (Mendes et al., 2018), barley (Bulgarelli et al., 2015) and *Arabidopsis thaliana* ssp. (Lundberg et al., 2012). We also observed an increase in the richness and diversity in the sunflower rhizosphere that positively correlated to the level of resistance to SVW of the inbred lines here studied. However, this correlation was not statistically significant ($P > 0.05$), indicating that only a trend could be inferred. Previous studies have also reported increases in alpha diversity, associated with increased resistance to pathogens (Masenya et al., 2021; Mendes et al., 2018; Zeng et al., 2022).

The structure of the sunflower rhizosphere bacterial communities was affected primarily by the genotype. Despite the observed shifts in the communities upon challenging with *V. dahliae*, the structure of the belowground bacteriome was not significantly altered. An important result of the present study is that differences in the sunflower

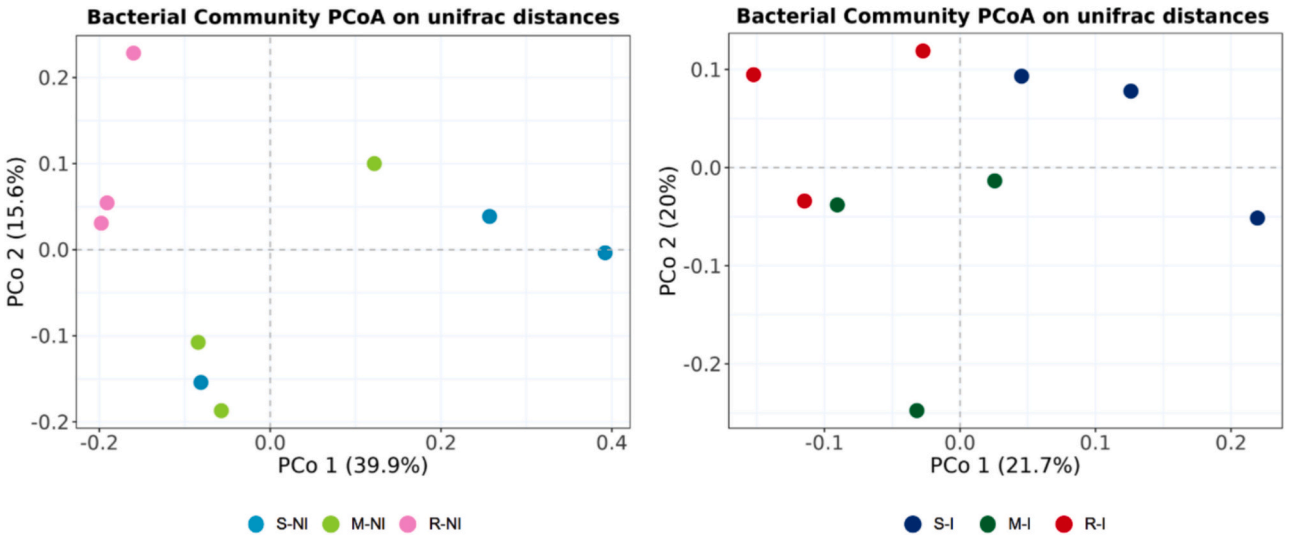

Fig. 2. Principal Coordinates Analysis (PCoA) based on UniFrac distances of the bacterial communities present in the rhizospheres of non-inoculated (NI) **(A)** and inoculated (I) sunflower inbred lines **(B)** with *Verticillium dahliae.* S: susceptible; M: moderate; R: resistant.

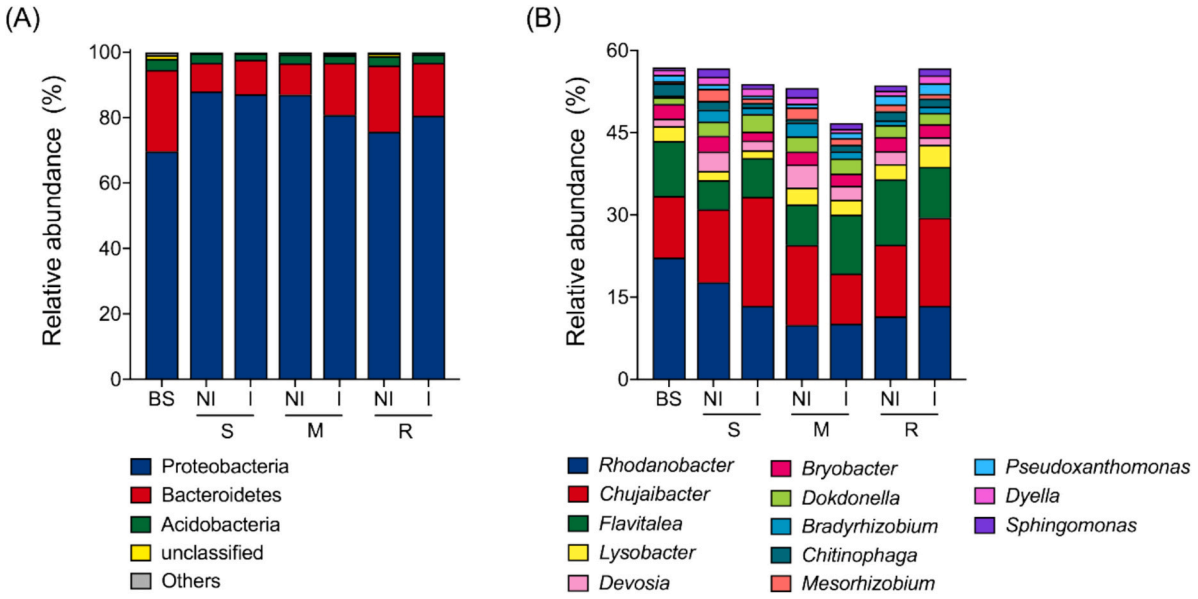

Fig. 3. Taxonomic profiles of the bulk soil and rhizosphere bacterial communities of different sunflower genotypes at phylum **(A)** and genus **(B)** levels. Phyla representing <1 % of the average relative abundance were grouped into "Others" group. Only genera representing >1 % of the average relative abundance are displayed. NI: non-inoculated; I: inoculated with *Verticillium dahliae*; BS: bulk soil; S: susceptible; M: moderate; R: resistant genotypes.

rhizosphere bacteriome were much greater among genotypes than those observed between non-inoculated and *V. dahliae*-inoculated plants within the same inbred line. Thus, our results do not support the second hypothesis of this work. The variances in the rhizosphere bacteriome among inbred lines displayed genotype-dependent selection, a phenomenon observed in other plant species infected with soil-borne pathogens (Fernández-González et al., 2020; Hassani et al., 2023; Lazcano et al., 2021; Zeng et al., 2022).

Interestingly, the three most abundant phyla found across all samples, namely Proteobacteria, Bacteroidetes and Acidobacteria, corresponded to those previously reported in the sunflower rhizosphere (Alawiye and Babalola, 2021; Nwachukwu et al., 2023; Pogoda et al., 2023). The relative abundance of most bacterial phyla was lower in the rhizosphere compared to the bulk soil, except for Proteobacteria, a trend that has been consistently observed in various plant species (Lundberg et al., 2012; Peiffer et al., 2013) including the strawberry rhizosphere infected with *V. dahliae* (Lazcano et al., 2021).

At genus level, high abundance of *Rhodanobacter*, *Chujaibacter*, *Flavitalea, Lysobacter, Devosia, Bryobacter, Dockdonella*, and *Chitinophaga* was found. Remarkably, just one ASV identified as *Chujaibacter* accounted for 9–19 % of the total sequences. Meanwhile, *Rhodanobacter* (two ASVs) represented 7.3–16 % and the relative abundance belonging to *Flavitalea* was 3.5–5.7 %. This dominance of a small number of ASVs could explain the lower Evenness observed for the rhizosphere samples. Furthermore, these most abundant genera, with the exception of *Lysobacter*, were identified as part of the core bacteriome and accounted for 38 % of the total sequences.

In addition, different accessory bacteriomes corresponding to each genotype were identified. Interestingly enough, the composition of these cores changed upon inoculation with *V. dahliae*. On the one hand, the core plant microbiota is widely accepted as a crucial for the fitness and growth of the host, as well as an important defense line against

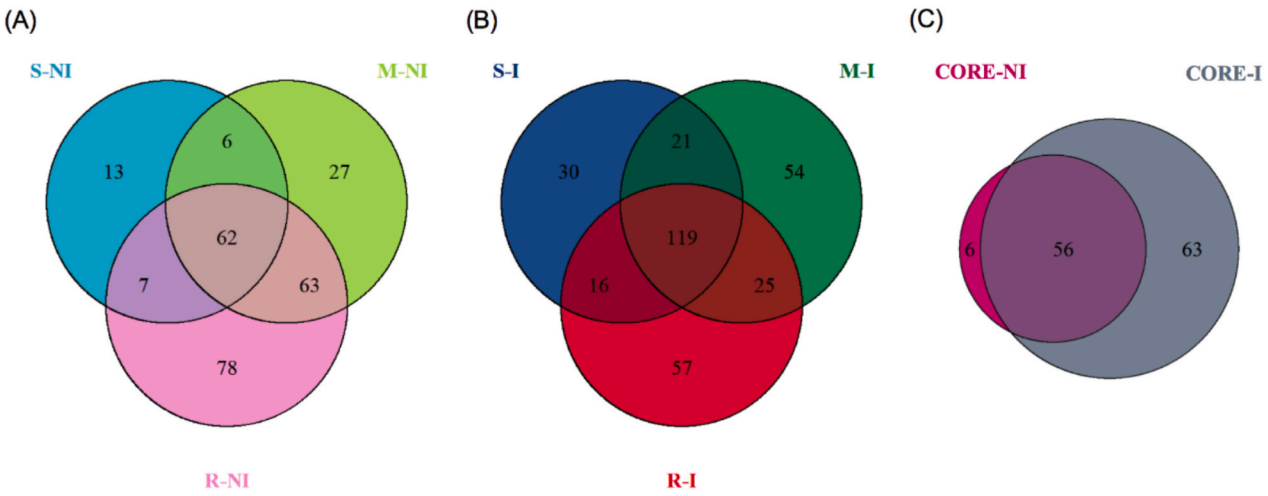

Fig. 4. Venn diagrams depicting the number of unique ASVs and shared ASVs among the non-inoculated **(A)** and *V. dahliae*-inoculated three sunflower genotypes **(B)**. **(C)** Core bacteriome of non-inoculated and *V. dahliae*-inoculated plants compared to each other. Each circle in the Venn diagram represents one genotype [(or the shared ASVs among genotypes, (**C**)], and the numbers inside the circles indicate both the unique and shared ASVs between genotypes. NI: non-inoculated; I: inoculated; BS: bulk soil; S: susceptible; M: moderate; R: resistant.

pathogens affecting the plant holobiont (Berg et al., 2020; Compant et al., 2019; Dong et al., 2022; Trivedi et al., 2020). On the other hand, the accessory bacteriome is supposed to encompass minor functions provided by specific microorganisms whose presence is influenced by interactions with the environment (Abera et al., 2022; Vandenkoornhuyse et al., 2015; Vos, 2023). It is tempting to speculate that the accessory cores belonged to each inbred line could play a role in the resistance to SVW. Finally, it is worth noting that we have been able to determine the sunflower core bacteriome, regardless of the genotype and the presence of a pathogen. Differential abundance analysis was conducted to assess whether specific taxa could be differentially recruited by the sunflower genotypes examined here, both in control and *V. dahliae*-challenged conditions. Indeed, several bacterial taxa increased their relative abundances in the different inbred lines and compared to bulk soil. This finding is consistent with previous studies reporting the recruitment of significantly different microbial communities by diverse plant species growing in similar soil environments, both in the rhizosphere and the root interior (Compant et al., 2019; Park et al., 2023). Furthermore, the presence of *V. dahliae* produced a shift in the abundance of certain taxa. Different studies suggest that *V. dahliae*, as well as the molecular dialogue established with the host plant, lead to crucial changes in the rhizosphere microbiome structure (Durán et al., 2018; Fernández-González et al., 2020; Tie et al., 2023). These changes provide evidence on the role of bacterial taxa in the "Cry for Help" strategy, according to which, in response to pathogen attack, plant roots initiate the recruitment of a large number of plant-associated microbes for disease suppression from the untapped soil reservoir, particularly beneficial bacteria (Li et al., 2021; Park et al., 2023; Rolfe et al., 2019).

In the rhizosphere of the *V. dahliae*-inoculated resistant inbred line (PMA26), significant changes in the abundance of *Lysobacter* and *Dyella* were observed when comparing with the non-inoculated plants. Both genera belonged to the core of the inoculated plants and, interestingly enough, two additional unique ASVs of *Lysobacter* were also identified as part of the R-I accessory core. The genus *Lysobacter* has been previously reported as effective suppressor of plant diseases through the production of a myriad of secondary metabolites with antimicrobial properties (Bejarano et al., 2021; Dastogeer et al., 2022; Gómez Expósito et al., 2015; Wei et al., 2019a, 2019b). On the other hand, *Dyella* has been associated with the suppression of wheat diseases (Yin et al., 2013), and was identified as a keystone genus associated with the suppression of *Ralstonia solanacearum* in tomato plants (Wei et al., 2019a, 2019b). A decrease in the relative abundance of *Dyella* was observed in the rhizosphere community of the moderate genotype under study. Interestingly, Cardoni et al. (2023) also reported a reduction in the relative abundance of this bacterium in the roots of the *V. dahliae*-susceptible olive cultivar `Picual´ when inoculated with this pathogen.

The bacterial community composition of the *V. dahliae*-moderate inbred line (RHA439) showed that *Flavobacterium* and *Flavisolibacter* were significantly enriched in the rhizosphere when these plants were inoculated with the pathogen. These genera are well-known as plant growth-promoting rhizobacteria (PGPR) and biocontrol agents (BCA), being effective even against *V. dahliae* (Berg, 1996; Bonanomi et al., 2018; Di Benedetto et al., 2017; Gallego-Clemente et al., 2023; Kwak et al., 2018; Lazcano et al., 2021; Liu et al., 2018; Mayak et al., 2004; Yin et al., 2020; Zeng et al., 2022). These results suggest that *Lysobacter* and *Dyella* in the R-I rhizosphere and *Flavobacterium* and *Flavisolibacter* in the M-I rhizosphere may play a role in the control of *V. dahliae*.

Nine genera (*Edaphobaculum*, *Variibacter*, *Ferruginibacter*, *Thermomonas*, *Rhizomicrobium*, *Pseudolabrys*, *Phenylobacterium*, *Micropepsis* and *Altererythrobacter*) were observed to increase significantly following the inoculation of the susceptible inbred line (RHA266) with *V. dahliae*.- Members of these genera have previously been positively correlated with soil health or regarded as effective plant growthpromoting bacteria and biocontrol agents against different pathogens affecting important crops (Duan et al., 2023; Huang et al., 2020; Lazcano et al., 2021; Li et al., 2023; Qi et al., 2020; Todorović et al., 2023; Sun et al., 2014; Wei et al., 2020; Wu et al., 2021; Ye et al., 2023; Yim et al., 2015; Zheng et al., 2024).

These findings may suggest that introduction of *V. dahliae* modifies the production of plants exudates in the S—I inbred line that resulted in an enrichment, from the bulk soil, in beneficial bacterial taxa able to stimulate the plant's growth and suppression of fungal infection as a mechanism of Cry for Help. Indeed, after the inoculation, the bacterial profile of S—I tends to be similar to those of the inoculated M and R inbred lines.

The ANCOM-BC analysis also revealed a decrease in the relative abundance of the genera *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Afipia*, *Devosia*, *Sphingomonas, Novosphingobium*, and *Altererythobacter* in the inbred lines upon inoculation with *V. dahliae*. Snelders et al. (2020) reported that the secreted effector VdAve1 from *V. dahliae* has antimicrobial activity and the ability to modulate rhizosphere microbiome composition. Furthermore, they observed that *V. dahliae* and the VdAve1 effector strongly affected the abundances of *Sphingomonas*, *Novosphingobium*, *Altererythobacter,* and *Rhizobium* in the tomato and

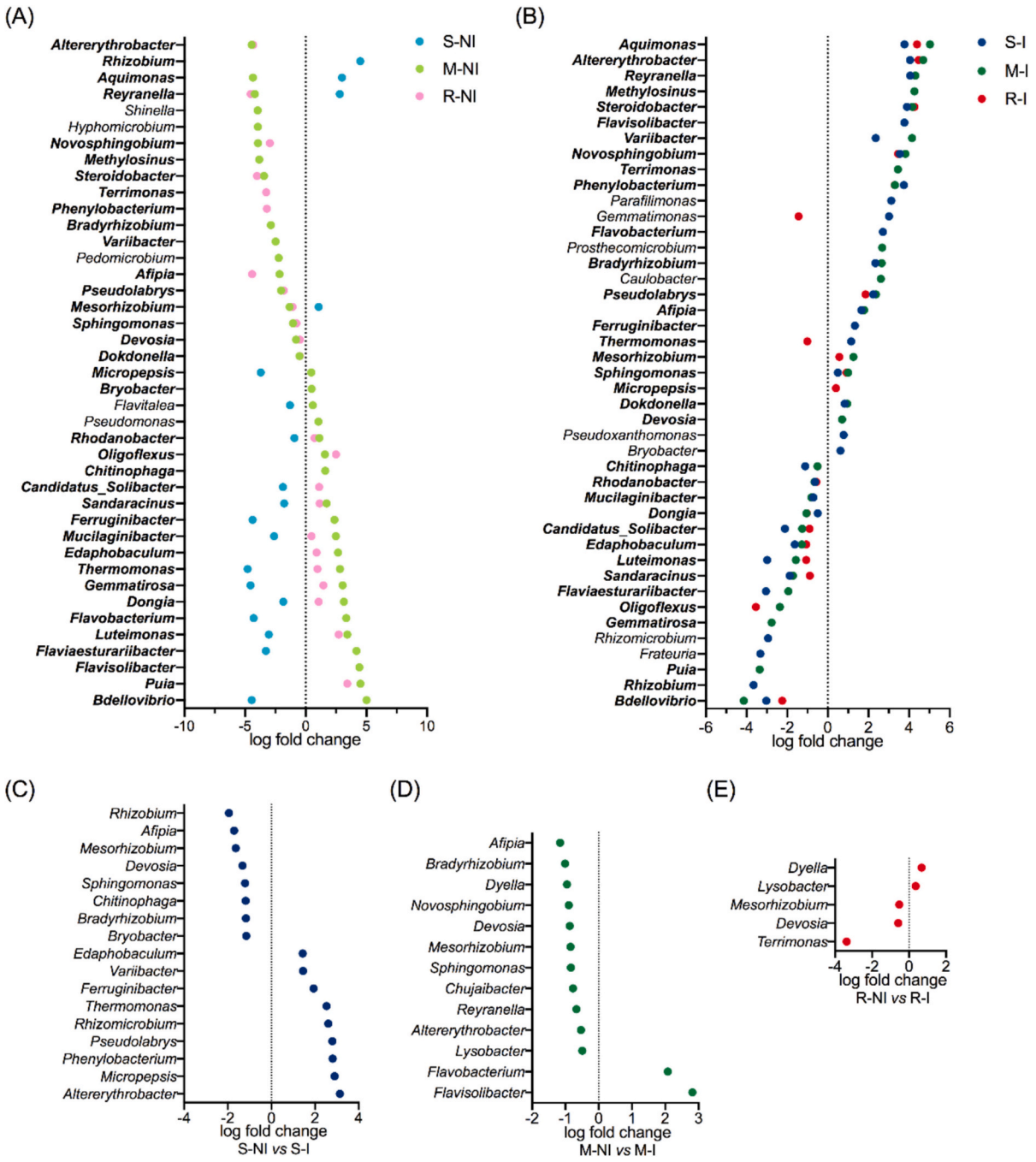

Fig. 5. Differences in the abundance of bacterial genera according to Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC). Bulk soil vs. rhizosphere of non-inoculated **(A)** and Bulk soil vs. rhizosphere of *Verticillium dahliae*-inoculated plants **(B)**. S-NI vs. S—I rhizosphere (**C**), M-NI vs. N—I rhizosphere (**D**) and, R-NI vs. R-I rhizosphere (**E**). Genera shown had statistically significant differences in their abundance ($P < 0.05$). Genera in bold letters are shared in (A) and (B). In (A) and (B) a positive log fold change value indicates that the relative abundance of the corresponding bacterial genera is higher in the sunflower rhizosphere than in bulk soil. In (C), (D) and (E) a positive log fold change value indicates genera enriched in the *Verticillium dahliae*-inoculated rhizosphere than the in non-inoculated rhizosphere. S: susceptible; M: moderate; R: resistant genotypes; NI: non-inoculated; I: inoculated.

cotton microbiomes. Likewise, Lazcano et al. (2021) described a reduction in the relative abundance of *Bradyrhizobium* and *Novosphingobium* in the rhizosphere of strawberry plants inoculated with *M. phaseolina*. Remarkably, we observed a similar pattern in the rhizosphere of sunflower. In the case of *Devosia*, in contrast to our results, other studies reported higher abundance in the rhizosphere of diseased plants (Becker et al., 2023), suppressive soils to banana wilt and nematodes and olive roots infected with *V. dahliae* (Martí et al., 2020; Todorović et al., 2023; Topalovic et al., 2020). Interestingly enough, Fernández-González et al. (2020) found that members of the genera *Devosia* and *Rhizobium* were keystones in the root endosphere microbiome of the *V. dahliae*-tolerant olive cultivar 'Frantoio' when inoculated with this pathogen.

According to these findings, the genotype strongly determined the bacterial communities of the sunflower rhizosphere. These results showed that independent of the sunflower resistance phenotype, the different inbred lines assemble a distinct bacteriome from the bulk soil. The highly susceptible sunflower inbred line RHA266 assembles a distinct rhizosphere bacteriome from that of moderate RHA439 or resistant PMA26 inbred lines. We want to stress that none of the

sunflower inbred lines showed significant differences in the bacterial community when comparing non-inoculated and *V. dahliae*-inoculated plants. Nevertheless, the rhizosphere bacterial communities showed significant changes in the relative abundance of some taxa, likely explained by *V. dahliae* inoculation. The bacterial rhizosphere communities of the moderate and resistant inbred lines were not significantly altered by *V. dahliae*, which suggests that sunflower plants assemble a rhizosphere bacteriome that is resistant to fungal perturbation. In contrast, the susceptible inbred line rhizosphere bacterial community underwent major alteration upon pathogen inoculation.

In summary, the results of this study demonstrate the significant contribution of genotype in shaping the bacterial community inhabiting the rhizosphere of the examined sunflower inbred lines. Both differential abundance and bacteriome core analysis highlight a range of bacterial taxa that have been reported to provide beneficial effects on plant growth including disease suppression, antifungal properties, and plant growth promotion.

5. Conclusions

Our findings suggest that the microbial basis of resistance is host-dependent, and that specific genotype resistance factors play a relevant role in recruiting beneficial bacteria that can counteract pathogens. However, further comprehensive integrated omics studies involving diverse plant genotypes with different levels of resistance, as well as field conditions, are necessary to fully understand the mechanisms involved in recruiting a suppressive root microbiome. Unraveling these mechanisms will be crucial for the success of future plant breeding programs and for ensuring sustainability in agriculture.

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CRediT authorship contribution statement

Emiliano Ben Guerrero: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Ana V. Lasa:** Writing – review & editing, Software, Investigation, Formal analysis, Data curation. **Pablo Aguilera:** Methodology, Investigation. **Antonio J. Fernández-González:** Writing – review & editing, Software. **María Carolina Martínez:** Writing – review & editing, Methodology, Investigation. **Jesús Mercado-Blanco:** Writing – review & editing. **Manuel Fernández-López:** Writing – review & editing, Supervision. **Norma Paniego:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

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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.

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Data availability

Sequence data reported in this paper are available on the NCBI Sequence Read Archive (SRA) BioProject under the accession number PRJNA1137565.

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