Changes in rice rhizosphere and bulk soil bacterial communities in the Doñana wetlands at different growth stages

Macarena Iniesta-Pallarés [a], Manuel Brenes-Álvarez [a], Ana V. Lasa [b], Manuel Fernández-López [b], Consolación Álvarez [a], Fernando P. Molina-Heredia [a], Vicente Mariscal [a,*]

[a] *Instituto de Bioquímica Vegetal y Fotosíntesis, Consejo Superior de Investigaciones Científicas and Universidad de Sevilla, Américo Vespucio 49, 41092 Seville, Spain*
[b] *Departamento de Microbiología del Suelo y la Planta, Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, 18008 Granada, Spain*

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ABSTRACT

The Doñana wetlands comprise an emblematic Mediterranean landscape protected as a UNESCO World Heritage Site. Some parts of these wetlands have been transformed into intensive rice cultivation areas, which are currently the most productive rice-growing areas in Europe. We examined the bacterial communities in these domesticated soils as they are key for plant health and productivity and have a strong influence on biochemical cycles. To identify the bacteria, we used metabarcoding analysis coupled with metabolic predictions and co-occurrence networks. This analysis was performed in the bulk and rhizosphere soils during different stages in the growing season. These soil compartments had a greater effect on the bacterial communities than the plant phenological stages. The diversity and richness of the bacterial population inhabiting the rhizosphere was much lower than that in the bulk soil, comprising taxa that were significantly more represented in this soil compartment, such as bacteria from the genus *Hydrogenophaga*, three genera from the order Rhizobiales, and unclassified genera from the families Desulfocapsaceae and Actinobacteria. Rhizosphere co-occurrence networks revealed a high number of negative connections, indicating unstable bacterial communities that may be highly influenced by biotic and abiotic factors. Rhizosphere networks mostly rely on two taxa belonging to the phyla Proteobacteria and Cyanobacteria, which are the predicted network hubs in this soil compartment. The bulk soil conserved high bacterial diversity and richness that was stable throughout the growth period of rice. Anaerobic bacteria from genera *Marmoricola*, the uncultured Gemmatimonadota bacteria SDR1034 terrestrial group, *Anaerolinea*, and the sulphur oxidizer, *Thiobacillus* were highly represented. This analysis provides valuable information for understanding bacterial diversity in the rhizosphere of rice cultivated in this region, which is critical for enhancing plant growth and productivity.

1. Introduction

Soil microbiota play an essential role in ecosystem function, they drive the Earth's biogeochemical cycles, and determine the availability of many essential plant nutrients, such as nitrogen, phosphorus, and carbon, among others (Domeignoz-Horta et al., 2020; Scarlett et al., 2021). Soil microbial interactions are crucial for ecosystem restoration and benefits (Lin et al., 2021). However, intensive farming practices, including excessive addition of nutrients and phytochemicals, reduces soil biodiversity, negatively impacting the interaction networks between functional communities resulting in fewer functional groups of soil biota with positive interactions (Tsiafouli et al., 2015; Huang et al., 2019; Wu et al., 2021). Additionally, soil biodiversity loss is associated with the

proliferation of opportunistic microorganisms, some of which are soil-borne plant pathogens that reportedly arise from conventional intensive agriculture (Ali et al., 2017). One such area is the Andalusian paddy fields which comprise 40,000 ha of wetland that has been transformed into arable soil for rice production (Aguilar and Borjas, 2005). Intensive cultivation practices have been used in this region, including continuous flooding, nutrient addition, and sowing high-yield plant varieties (Aguilar and Borjas, 2005). In addition, the Guadalquivir River-sourced irrigation water is reused in many plots, concurrently supplying phytosanitary products and increasing water salinity (Aguilar et al., 2017). However, the diversity and structure of soil microbial communities in these transformed wetlands has not been sufficiently investigated.

In nature, plants are constantly challenged by thousands of different

microbial populations, including commensals, pathogens, and symbionts (Ali et al., 2017). Plants not only rely on but also shape the microbial community structure inhabiting the soil through their root exudates (Chaparro et al., 2013; Korenblum et al., 2020). This local system of plant roots, soil, and resident microbial communities is known as the rhizosphere. Bacterial communities in rice rhizospheres are diverse and dynamic and they are dependent on plant type, soil conditions, and nutrient availability (Edwards et al., 2015). Rhizosphere microbial communities help increase host tolerance to biotic and abiotic stresses, improve adaptation to environmental variations, and enhance nutrient acquisition (Bulgarelli et al., 2013; Castrillo et al., 2017; Hassani et al., 2018). Therefore, plant fitness is a consequence of the plant itself and its microbiota, which collectively form the plant holobiont (Vandenkoornhuyse et al., 2015). Consequently, the rhizosphere microbiota is typically referred to as a second or extended plant genome (Berendsen et al., 2012).

The developmental stage of plants can significantly influence the bacterial community of their rhizosphere, with changes in bacterial diversity, abundance of specific bacterial groups and changes in root exudation patterns observed throughout the different growth stages (Edwards et al., 2015; Das et al., 2018). Changes in the bacterial community composition have been found in the rhizosphere of crop plants at different phenological stages. For instance, in rice, bacterial taxa such as *Pseudomonas* spp., *Bacillus* spp., *Rhizobium* spp. and *Comamonas* spp. are highly prevalent in the reproductive stage (Rasche et al., 2006; Edwards et al., 2015). The microbial community in different growth stages of other crops such as pea, wheat, sugar beet, and tomato also vary with the phenological stage, and are most different between the vegetative and flowering stages of pea, sugar beet, and tomato plants, and between the vegetative and senescence stages of wheat (Houlden et al., 2008; Ofek-Lalzar et al., 2014).

Crop management practices impact root microbial communities, which may negatively impact plant productivity. Several studies have reported that long-term agricultural management decreases the number of beneficial interactions between plants and microbes, negatively impacting plant biotic and abiotic stress resistance (Jacoby et al., 2017; Huang et al., 2019; Tao et al., 2019). Paddy fields are usually cultivated following Green Revolution practices, which involve the extensive use of nitrogen-rich fertilisers, improved irrigation facilities, flooding maintained for an entire season, and the use of high-yield varieties of rice (Khush, 2001). Cultivation under flooding conditions lead to a strong anoxic microbial activity in the deeper layers of the soil, with a prominent biogenic source of methane from microbial activity (Bao et al., 2016). Bacterial activity related to anoxic denitrification has been frequently reported in fertilised paddy soils (Ishii et al., 2011; Zhu et al., 2011). Thus, bacteria belonging to the orders Burkholderiales (especially the genus *Herbaspirillum*), Rhodocyclales (genus *Dechloromonas*), and Rhizobiales have been commonly detected in rice paddy soils under denitrification-inducing conditions (Ishii et al., 2011; Hara et al., 2019).

This study was conducted in an Andalusian paddy field and data on the bacterial communities inhabiting these paddies are limited. Therefore, we hypothesised that plant phenological growth stage determines the abundance and diversity of bacterial communities in the rhizosphere, that have been selected from bacterial communities in the bulk soil. The experimental plot is representative of the environmental status of this protected area, which has been used for rice cultivation for >40 years. In this study, we addressed the following questions: (1) What is the structure, functionality, and stability of the bulk soil and rhizosphere bacterial communities in in this anthropogenic wetland? (2) How does the soil bacteriome change with plant phenologycal stage? (3) Which microorganisms are key to supporting the stability of microbial communities in bulk and rhizosphere soils? To answer these questions, we studied the diversity, structure, taxonomical profiles, potential functionality, and assembly of the prokaryotic communities in bulk and rhizosphere soils during the crop season using metabarcoding techniques targeting the *16S rRNA* gene. Our findings highlight ways

through which intensive cultivation can impact microbiological community structures, and can be used in rice cultivation techniques beyond those currently used in Andalusian rice paddies.

2. Materials and methods

2.1. Site description and sample collection

The experimental site is located in the Doñana wetlands (southwest Spain). The location constitutes 180,000 ha of marshland in the Guadalquivir delta, containing 40,000 ha devoted to rice cultivation. Its proximity to the Atlantic Ocean, moderates temperatures and increases humidity producing a mild and temperate climate with an average annual temperature and precipitation of 18 °C and 600 mm, respectively.

In contrast to this natural area, the northern sector, which was developed in the 20th century, has become a more anthropogenic environment. Thus, the long-term agricultural use in this area must be considered as it has undergone intensive irrigation for rice fields and other herbaceous crops. Rice paddies are irrigated with water from the Guadalquivir River and maintained with continuous flooding for the entire growing season. This detail is critical as paddies require a large N supply to increase their production. Generally, paddy fields are fertilised with 315 kg of urea per hectare annually.

For bulk soil analysis, 14 soil samples were collected in sterile containers from seven different locations across the Doñana paddy fields in March 2018 (Supplementary Fig. S1). The GPS coordinates of the different plots used are listed in Supplementary Table S1. In each plot, five representative soil samples taken equidistantly were pooled into a single composite sample. Two composite samples were obtained from each location. They were then transported to the laboratory under refrigerated conditions, immediately frozen in liquid nitrogen, and stored at −80 °C until use.

Samples from plot PC18 (Supplementary Fig. S1) were collected for in-depth analysis of the bacteriome throughout the planting and heading time because of its location and soil homogeneity. Four replicates of soil and plant samples, which were uprooted and conserved at 4 °C, were collected at three different times after planting: 1 month (vegetative stage), 2 months (reproductive stage), and 4 months (ripening stage). For the selection of the different phenological stages, the development of the main stem was considered. Thus, as representative for the vegetative stage, plants with three leaves (16.5 ± 2.1 cm in length) were selected. For the reproductive stage, plants with an initiated panicle, with three to five tillers (37.4 ± 2.8 cm in length) were selected. For the ripening stage, plants with visible panicles (67.3 ± 6.0 cm in length) containing mature grains were selected. Twelve each of bulk soil and rhizosphere samples were obtained from this plot and transported to the laboratory; the bulk soil samples were immediately frozen at −80 °C. Rice roots were washed with sterile water to remove unattached soil, and roots with attached soil were frozen at −80 °C.

2.2. Soil analysis

A fraction of each sample was air-dried and sieved using a 2-mm sieve for chemical analysis. Soil pH was measured in a 1:2.5 suspension ratio of soil to KCl solution (1 M) after 30 min of shaking. N was determined following the Kjeldahl method using an automatic distillation system with titration (Vapodest 50S, Gerhardt). Total NO_3^-, NH_4^+, and P (Olsen's procedure) were determined using an automatic segmented flow analyser (Bran-Luebbe). X-ray fluorescence spectroscopy (XRF Niton) was used to measure total soil cations (Ca, K, Zn, Cu, Fe, Mg, and Mn).

2.3. DNA extraction and Illumina sequencing

For bacteriome analysis, DNA was extracted with the NucleoSpin Soil

Kit following the manufacturer's instructions. After quantifying and analysing DNA quality, the samples were subjected to Illumina amplicon sequencing using Eurofins Genomics Services (Ebersberg, Germany). The V4–V5 hypervariable regions in the bacterial *16S rRNA* gene were sequenced in all 38 samples from the bulk and rhizosphere soils. The universal primers F515 (5′-GTGYCAGCMGCCGCGGTAA-3′) and R907 (5′-CCGTCAATTCMTTTRAGTTT-3′) (Zheng et al., 2018) were used, and a paired-end 2 × 300 bp sequencing strategy was followed.

2.4. Bioinformatic processing of sequencing data

In total, 5,913,472 raw sequences were obtained from the 38 samples with an average of 155,618 reads per sample. Pre-processing and quality control filtering, operational taxonomic unit (OTU) picking, and taxonomy assignment were performed using the Galaxy Europe Server https://usegalaxy.eu/ (Afgan et al., 2018). These analyses were conducted using the mothur project tools (Schloss et al., 2009; Schloss, 2020). The pipeline used was based on the method described by Kozich et al. (2013) (also described in https://mothur.org/wiki/miseq_sop/). If no parameters are specified, default parameters were used in the downstream analysis.

Paired-end reads were merged using the "*make.contigs*" function, and sequences were trimmed according to the length of the amplified fragment (372 bp) using the "*screen.seqs*" function. Fragments were aligned against the SILVA v138 database using the "*align.seqs*" function (Balvočiute and Huson, 2017; Glöckner et al., 2017). Sequences aligning to the V4–V5 rRNA region were trimmed and kept for further analysis ("*filter.seqs*" and "*screen.seqs*" functions). Sequences differing by 1 out of 100 nucleotides were pre-clustered ("*pre.cluster*" function). Sequences containing homopolymers with more than eight nucleotides, ambiguous base calls, and chimeric sequences were removed using the VSEARCH algorithm (Rognes et al., 2016). After these analyses, 3,965,694 high-quality sequences remained.

After a taxonomical classification against the SILVA v138 database (using the "*classify.seqs*" function with default cut-off value), sequences classified as chloroplasts, mitochondria, archaea, eukaryotes, and unknown sequences, were removed from the analysis. To improve the computational analysis, we discarded sequences (using the "*split.abund*" function) that appeared <10 times in all samples, as they were considered rare sequences. After all the trimming and filtering steps, 2213 OTUs were retained for further analysis. Filtered sequences were taxonomically classified using the SILVA v138 database and the "*classify.seqs*" function. After taxonomical classification, sequences were clustered into OTUs with 97 % genetic similarity using the "*cluster.split*" function. Considering the degree of bacterial taxonomy assignment, 100 %, 98.6 %, 88.6 %, 76.8 %, and 42.2 % of the OTUs were classified at the phylum, class, order, family, and genus levels, respectively.

We used the "*get.oturep*" function to select the representative sequence of each OTU based on its abundance. Phylogenetic distances between representative sequences were calculated using the "*dist.seqs*" function. A phylogenetic tree was inferred using clearcut (Sheneman et al., 2006). This phylogenetic information was later used for beta-diversity analysis.

2.5. Analysis of alpha and beta diversity

All statistical analyses were performed in R software using base function and package "*MicrobiomeAnalyst*" (Dhariwal et al., 2017; Chong et al., 2020) and "*vegan*", respectively (Oksanen et al., 2022). The webservers MicrobiomeAnalyst (www.microbiomeanalyst.ca) and METAGENassist (www.metagenassist.ca) were also used (Arndt et al., 2012).

Initially, we filtered OTUs based on their prevalence. OTUs with fewer than 10 reads in <10 % of samples were discarded. To remove potential biases related to different sequencing depths, a rarefaction step was performed on 42,317 random reads per sample, which is the lowest library size of the entire dataset. This rarefaction step was performed using the "*rarefaction.single*" function.

To estimate the alpha diversity (α-diversity) of the bacterial communities, the following ecological indices were calculated: observed and estimated OTU richness (Chao-1 index), Shannon and evenness, ACE, Simpson, and Inverse Simpsonindices. Diversity index distributions were tested for normality and homogeneity of variances. A Mann–Whitney test was conducted for non-normal distribution. For normal distribution, ANOVA was used for multiple comparisons, and Student's *t*-test was used as a *post-hoc* test to determine if there were significant differences between the means of two groups. In all tests, a *p*-value <0.05 was considered statistically significant.

Beta diversity (β-diversity) analyses were performed to compare the structure of the total bacterial community between samples, that is, the compositional dissimilarity between communities. Weighted UniFrac (Lozupone and Knight, 2005) was selected as the distance metric for the β-diversity analyses and was calculated using the "*phyloseq:distance*" function (McMurdie and Holmes, 2013). Permutational analysis of variance (PERMANOVA) was used to test the differences between sample groups using the "*vegan:adonis*" function (Oksanen et al., 2022). Sample grouping along the multivariate space was performed with principal coordinate analysis (PCoA) using the MicrobiomeAnalyst tool. The "*ANCOM2*" package (Mandal et al., 2015) was then used to perform differential abundance analysis at all taxonomic levels. A conservative detection threshold for differentially abundant taxa of 0.8 was chosen, and *p*-values were corrected using the Benjamini–Hochberg method for multiple comparisons.

2.6. Prediction of functional profiles based on bacterial taxonomy

A rarefied OTU table was used to predict bacteriome functions using METAGENassist, which matches the taxonomic data with the phenotype from a database containing phenotypic information of 11,000 or more bacterial and archaeal species listed in the National Center for Biotechnology Information (NCBI) microbial taxonomy database (Arndt et al., 2012). Input data were normalised by sum. Raw values of metabolic functions provided by the program were converted to percentage.

2.7. Co-occurrence networks

Bacterial networks were individually calculated for the bulk and rhizosphere soils, considering all replicates in each case (N = 12 as the total rhizosphere samples and N = 11 as the total bulk soil samples). A Molecular Ecological Network Analysis Pipeline (MENAp; Zhou et al., 2010; Zhou et al., 2011; Deng et al., 2012; http://ieg4.rccc.ou.edu/mena/main.cgi) was used for network calculation. Initially, a prevalence cut-off of 50 % was selected; thus, OTUs that were present in less than five replicates were eliminated from the analyses. Logarithmic transformation was applied to the abundance of the remaining OTUs, and subsequently, a similarity matrix based on Pearson's correlation coefficient was constructed. For the modularity calculation, fast-greedy optimisation of modularity was selected.

For each empirical network, 100 random networks were calculated, maintaining the number of nodes and links as the corresponding empirical network but changing their topology. To compare the global properties of the empirical networks, the Student's *t*-test was applied by employing the standard deviation of the corresponding randomised networks. Subsequently, Cytoscape software (version 3.8.2.0; Shannon et al., 2003) was used to construct co-occurrence network graphs. Nodes were classified as connectors, module or network hubs, and peripherals according to the description provided by Olesen et al. (2007), considering within- and between-module connectivity.

2.8. Availability of high-throughput sequencing data

The dataset obtained by high-throughput sequencing is publicly

available in the NCBI Sequence Read Archive (SRA) under the accession code PRJNA813511.

3. Results

3.1. Soil physicochemical analyses

Soil physicochemical analyses (Table 1) revealed relatively high conductance (1.411 ± 0.139 mS·cm^{-1}) and Na levels (1.198 ± 0.128 g·kg^{-1}), indicating soil salinisation. We also detected soluble N sources such as nitrate (5.720 ± 0.994 mg·kg^{-1}) and ammonium (8.050 ± 0.994 mg·kg^{-1}) in high proportions.

3.2. Bacterial composition and richness in Andalusian paddy soils after winter

First, we determined the bacterial richness and composition of paddy soils during winter (non-cultivating season), when paddy fields are not flooded and consist of upland field-like bacterial communities (Kirk et al., 2004). Thus, we analysed the bacteriome from the seven selected plots (Supplementary Fig. S1). PCoA based on weighted UniFrac distances revealed that the samples were not distinguished by their distribution regarding the irrigation channel (Supplementary Fig. S2A), which was supported by the PERMANOVA test (F = 1.248; p = 0.241). Thus, all samples were analysed together. All the diversity indices are listed in Supplementary Table S2. Members of the phyla Proteobacteria (24.2 ± 5.0 %) and Chloroflexi (23.4 ± 6.4 %) dominated the samples. Among them, the genera *Anaerolinea* (2.7 ± 0.8 %), SBR1031 (2.7 ± 1.3 %), UTCFX1 (2.4 ± 0.8 %), and the sulphur oxidiser, *Thiobacillus* (3.4 ± 1.7 %), were highly represented in these samples (Fig. 1A; Supplementary Table S3). Other genera from different phyla were also highly represented in the samples, including the anaerobic, *Marmoricola* (5.3 ± 4.0 %), and the uncultured Gemmatimonadota bacteria, SDR1034 terrestrial group (2.7 ± 1.8 %).

Second, we determined the metabolic potential of these soil bacteria. The taxonomic profiles were inputted into METAGENassist (Arndt et al., 2012). Regarding oxygen requirements, we found anaerobic (35.54 ± 5.77 %) and aerobic bacteria (24.47 ± 6.41 %). Of the 106 main metabolic activities included in the METAGENassist database, two functions related to the N cycle accounted for 40 % of those that registered. These metabolic functions were ammonia oxidation (22.26 ± 0.99 %) and nitrite reduction (18.81 ± 2.09 %) (Fig. 1B). Other metabolic activities indicated sulphur use, such as sulfate reduction (12.29 ± 1.01 %) and sulfide oxidation (11.63 ± 2.08 %), and pesticide degradation, such as dehalogenation (10.09 ± 1.65 %) or chlorophenol degradation (0.38 ± 0.12 %).

Table 1
Soil parameters in the Guadalquivir marshes paddies after the winter season. Mean and standard error from the different plots (n = 7) are shown. Phosphate (P), Potassium (K), Sodium (Na), Nitrite (NO$_3^-$), Magnesium (Mg), Calcium (Ca), Iron (Fe), Manganese (Mn), Zinc (Zn) and Copper (Cu).

pH	7.783 ± 0.039
Salinity (mS/cm)	1.411 ± 0.139
P (mg/kg)	11.390 ± 2.056
N Kjeldahl (%)	0.183 ± 0.105
K (g/kg)	0.668 ± 0.065
Na (g/kg)	1.198 ± 0.128
NO$_3^-$ (mg/kg)	5.720 ± 0.994
NH$_4^+$ (mg/kg)	8.050 ± 2.300
Mg (g/kg)	3.060 ± 0.398
Ca (g/kg)	17.231 ± 0.505
Fe (mg/kg)	184.773 ± 20.615
Mn (mg/kg)	74.269 ± 6.098
Zn (mg/kg)	2.286 ± 0.275
Cu (mg/kg)	10.651 ± 0.400

3.3. Differential bacteriome of rice rhizosphere and bulk soils

We explored the extent to which microbial communities changed in the bulk and rhizosphere soils. Thus, the sampling points in each soil type were analysed together. For α-diversity analyses, the bulk soil supported a higher Shannon diversity index value than that of the rhizosphere samples (Fig. 2A; Mann–Whitney test p = 1.5·10^{-6}). PCoA and PERMANOVA revealed that bulk soil and rhizosphere samples had different bacterial community structures (Supplementary Fig. S2B; PERMANOVA: F = 15.93; p = 9.99·10^{-5}). Some phyla were found in high proportions in both sample types, while others were only enriched in one (Fig. 2B; Supplementary Table S4). The differential abundance of bacterial taxa in the two compartments was calculated using *ANCOM2*. Notably, we found that 27 bacterial genera were overrepresented in the rhizosphere (Supplementary Table S5), in which the genus *Hydrogenophaga*, three genera from the order Rhizobiales (*Devosia, Ciceribacter*, and one unclassified), and unclassified genera from the families Desulfocapsaceae and Actinobacteria were surprisingly disproportionate in their abundance (Fig. 2C). In addition, we found 101 genera that had lower proportions in the rhizosphere than in the bulk soil, indicative of plant-driven bacterial selectivity. These diminished genera were *Thiobacillus, Marmoricola*, and an unclassified genus of the S0134 terrestrial group from the Gemmatimonadota phylum (Fig. 2C).

### 3.4. Changes in the soil bacteriome during cultivation

We explored the extent to which bacterial communities in the bulk and rhizosphere soils were affected during different cultivation stages: vegetative, maturation, and ripening. The PCoA plot showed that bacterial communities in the rhizosphere were affected by cultivation time (Supplementary Fig. S2C and D). This finding was supported by the PERMANOVA test (F = 2.655; p = 0.0058). We evaluated taxa with different abundances in the rhizosphere at different developmental stages using *ANCOM2* and identified 197 genera with a significant increase at specific time points (Supplementary Table S6). The most abundant genera with significant increases at each time point are shown in Table 2.

During the vegetative stage, the rhizosphere was dominated by aerobic microorganisms, of which the cyanobacterium, *Nodosilinea* PCC 7104, was the most abundant (20.10 ± 12.17 %). Other genera, including *Pseudomonas* (5.63 ± 4.67 %) and *Lewinella* (0.67 ± 0.52 %), were also detected at significant levels (Table 2). During the reproductive stage, the rhizosphere was enriched in heterotrophic microorganisms from the order, Myxococcales (6.29 ± 3.51 %), and genus *Halomonas* (4.35 ± 7.83 %), among others with low representation. The presence of halotolerant species such as *Marinoscillum, Parvularcula*, and *Flexibacter* in the rhizosphere during the vegetative and reproductive stages indicates the high salinity of the irrigation water, which recorded an average NaCl concentration of 1252 ± 0.009 g·L^{-1}. During the ripening phase, *Pleomorphomonas* spp. were the most abundant (1.54 ± 1.83 %). Different sulfate-reducing bacterial genera belonging to the family Desulfovibrionaceae were also identified during this stage (Table 2).

3.5. Co-occurrence networks of bacteria inhabiting the bulk and rhizosphere soils

Co-occurrence networks of the bulk and rhizosphere soils were constructed by considering all sampling points in each soil type together. They revealed that the association of bacterial communities inhabiting the bulk soil and the rhizosphere of rice plants were significantly different. As shown in Supplementary Fig. S3, the topology of both networks was slightly different; the network of the bulk soil samples was more compact than that of the rhizosphere samples. However, both networks were characterised by a modular structure, as the modularity (M) value was >0.4, indicating that both networks resemble

A

B
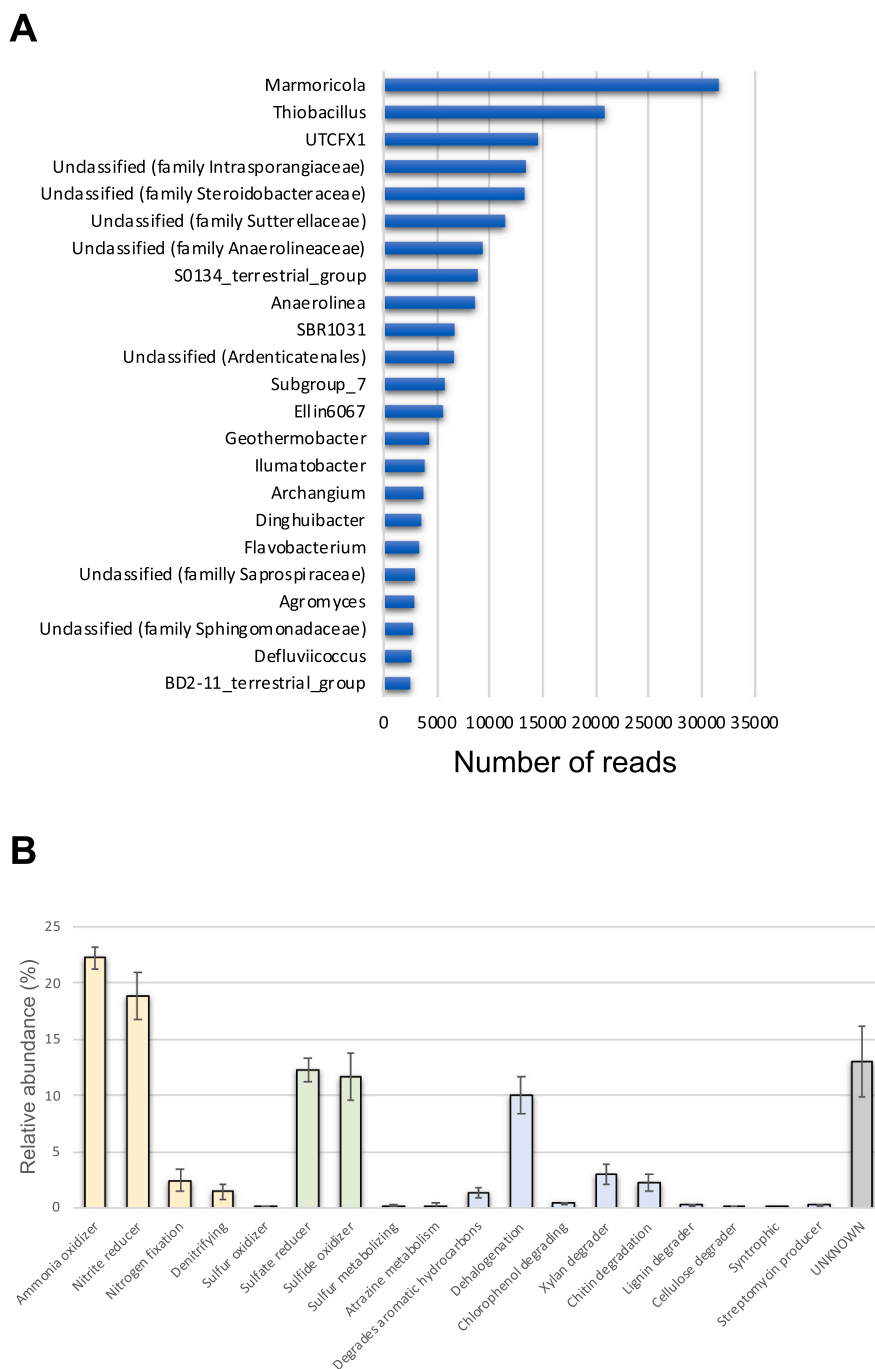
Fig. 1. Bacterial diversity and metabolic profiles of the soil bacteriome of paddy fields after winter. (A) Highly represented bacterial taxa from bulk soil samples based on the rarefied OTU table at a depth of 42,317 sequences per sample. (B) Prediction of the metabolic profiles of the bacteriome from bulk soil samples using the METAGENassist web service database.

small-world networks. Nevertheless, marked changes were observed in the global properties of the networks. Higher M and Distance (GD) values were recorded for the bulk soil samples (Table 3), which are usually associated with a stable and protective role in bacterial communities against disturbances. In contrast, both co-occurrence networks showed differences in complexity, indicated by high average degree (avgK) and clustering coefficient (avgCC) values. The rhizosphere network showed a high avgK value, and its associated avgCC value was significantly lower than that of the bulk soil (Table 3). Notably, the percentage of positive links was considerably higher in bulk soil than that in the rhizosphere network (Table 3 and Fig. 3). Considering the most representative modules in the bulk soil network, negative

interactions were mostly concentrated in module 5, although many negative links were found within and between other modules (Fig. 3A).

Importantly, different topological roles of network OTUs were found between the bulk and rhizosphere soils. In the rhizosphere, two OTUs were identified as network hubs, which were ascribed to phyla Proteobacteria and Cyanobacteria (Supplementary Table S7). However, no network hubs were detected in the bulk soil samples. They also differed in numbers and taxonomy of their module hubs. As summarised in Supplementary Table S7, 37 module hubs were detected in the rhizosphere, most of which were in modules 6 and 2. In the bulk soil, 59 module hubs were detected, five of which belonged to the SBR1031 order of the class Anaerolineae; however, none could be classified at the
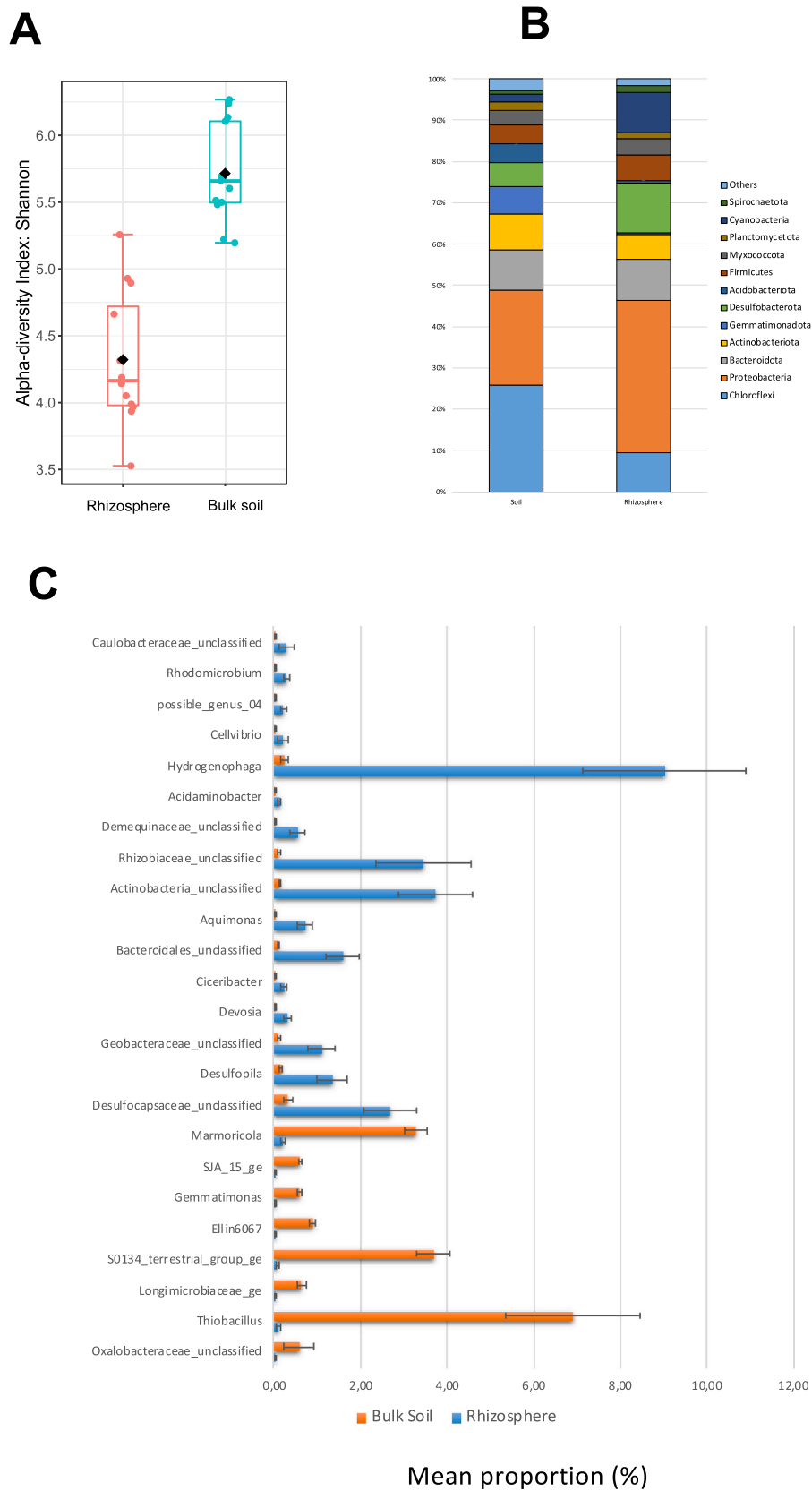
Fig. 2. Bacterial diversity in the bulk and rhizosphere soils during the cropping season. (A) α-diversity analysis of rhizosphere and bulk soil samples measured using the Shannon index. (B) Bar plot of the 12 main phyla found in rhizosphere and bulk soil samples using the rarefied OTU table at a depth of 42,317 sequences per sample. (D) Comparison of the 24 main genera differentially enriched in the rhizosphere and bulk soil samples based on the *ANCOM2* analysis.

Table 2

Main taxa overrepresented in the rice rhizosphere at vegetative, reproductive and ripening phases, which represented >1 % of the total sequences obtained. Figures, given in percentage, are the mean relative abundance of the corresponding taxa in each phase + − the standard deviation of the mean.

Genus	Vegetative	Reproductive	Ripening
Overrepresented during vegetative phase			
*Nodosilinea*_PCC_7104	20.10 ± 12.17	0.21 ± 0.10	0.07 ± 0.11
Pseudomonas	5.63 ± 4.67	0.65 ± 0.25	0.05 ± 0.07
Unclassified (family *Cyclobacteriaceae*)	1.35 ± 0.88	0.05 ± 0.01	0.003 ± 0.01
Lewinella	0.67 ± 0.52	0.005 ± 0.01	0.001 ± 0,002
Aeromonas	0.64 ± 0.84	0.11 ± 0.15	0.00 ± 0.00
*Phormidium*_MBIC10003	0.42 ± 0.18	0.001 ± 0.002	0.01 ± 0.02
Unclassified (family *leptolyngbyaceae*)	0.34 ± 0.22	0.00 ± 0.00	0.001 ± 0.001
Sandarakinorhabdus	0.28 ± 0.18	0.001 ± 0.001	0.00 ± 0.00
Unclassified (family *Saprospiraceae*)	0.22 ± 0.19	0.00 ± 0.00	0,03 ± 0.02
Fimbriiglobus	0.21 ± 0.16	0.01 ± 0.01	0.00 ± 0.00
Rheinheimera	0.20 ± 0.23	0.02 ± 0.04	0.00 ± 0.00
Cloacibacterium	0.20 ± 0.16	0.01 ± 0.01	0.00 ± 0.00
Overrepresented during reproductive phase			
Unclassified (order *Myxococcales*)	0.12 ± 0.16	6.29 ± 3.51	2.74 ± 1.31
Halomonas	0.02 ± 0.03	4.35 ± 7.83	0.00 ± 0.00
Psychrobacter	0.00 ± 0.00	0.64 ± 0.95	0.00 ± 0.00
Flexibacter	0.08 ± 0.07	0.44 ± 0.61	0.00 ± 0.00
Treponema	0.004 ± 0.004	0.21 ± 0.16	0.99 ± 0.80
Parvularcula	0.00 ± 0.00	0.14 ± 0.12	0.01 ± 0.02
Marinoscillum	0.00 ± 0.00	0.10 ± 0.10	0.005 ± 0.01
Overrepresented during vegetative and reproductive phase			
Leptonema	0.002 ± 0.003	0.18 ± 0.13	0.27 ± 0.13
Desulfocurvus	0.00 ± 0.00	0.11 ± 0.18	0.15 ± 0.19
Overrepresented during ripening phase			
Pleomorphomonas	0.03 ± 0.02	0.01 ± 0.03	1.54 ± 1.83
Unclassified (family *Desulfovibrionaceae*)	0.003 ± 0.01	0.12 ± 0.11	1.13 ± 0.65
Prevotella	0.05 ± 0.04	0.00 ± 0.00	0.27 ± 0.47
Lachnoclostridium	0.00 ± 0.00	0.00 ± 0.00	0.30 ± 0.51
*Bacteroidetes*_BD2_2	0.00 ± 0.00	0.07 ± 0.11	0.21 ± 0.23
Ignavibacteriales	0.00 ± 0.00	0.00 ± 0.00	0.15 ± 0.13
Unclassified (family *Chitinophagaceae*)	0.002 ± 0.004	0.00 ± 0.00	0.14 ± 0.11
Puniceicoccus	0.001 ± 0.001	0.00 ± 0.00	0.12 ± 0.19
Desulfomicrobium	0.00 ± 0.00	0.01 ± 0.01	0.11 ± 0.08

genus level.

4. Discussion

4.1. Bacterial diversity and composition of paddy soils

To the best of our knowledge, this is the first study to describe several ecological aspects of bacterial communities in Andalusian paddy fields and predict their functions. We found that bacterial composition was not affected by geographical distribution, and all plots contained the same community structure. These former wetlands have been devoted to rice cultivation and have been managed consistently for >40 years. Continuous ploughing and waterlogged conditions may have made the soil in this region more homogeneous, leading to relatively weak niche differentiation. Our findings also revealed the presence of putative new taxa in domesticated soils. Of the 5994 identified OTUs, 57.8 % did not match any taxa in the SILVA v138 database (updated in 2019). The families, Anaerolineaceae, Nocardioidaceae, and Hydrogenophilaceae, were the most abundant in the soil samples (Fig. 2B). We found 11 OTUs that belonged to the class Anaerolineae, with SBR1031 and UTCFX1 accounting for 2.4 % and 1.1 % of the total sequences, respectively, suggesting that these genera may have important roles in these soils. Other studies have reported a higher proportion of several genera of the Anaerolineaceae family in paddy soils than in non-paddy soils (Liechty et al., 2020; Li et al., 2021). Strains belonging to this family contain genes necessary for acetate production through anaerobic fermentation and use sugars derived from rice plants to produce acetate, CO_2, and H_2 (Sheng et al., 2013; Xia et al., 2016). Additionally, the activity and growth of this family are promoted by hydrogenotrophic methanogens. We also found a high proportion of *Marmoricola* and *Thiobacillus*, which may have significantly contributed to N and S cycles in soil, respectively (Fig. 1B). Members of the *Marmoricola* genus are chemoorganotrophic bacteria that have been found in marine sediments and volcanic ash and are relatively abundant in cultivated soils, where they have been associated with greenhouse gas production, and in some species, with respiratory metabolism and denitrification processes (Li et al., 2019; Tóth and Borsodi, 2014; Monreal et al., 2018; Lang et al., 2020). Members of the *Thiobacillus* genus are chemolithoautotrophic sulphur bacteria that are ubiquitously and abundantly found in soils. They play a significant role in the oxidisation of sulphur, by making it accessible to plants (Kelly and Wood, 1998).

From the 106 main metabolic activities predicted by the META-GENassist web server, activities related to the N and S cycles accounted for approximately 70 % of metabolism (Fig. 1C). Continuous fertiliser input containing urea for rice cultivation are likely selected for denitrifying communities, and the tight interactions between ammonia and nitrite oxidisers may be closely related to the physicochemical properties of the soil. Hence, these bacterial communities may be used as metabolomic biomarkers for monitoring and managing sustainable soil health, thereby providing an early sign of soil impoverishment or degradation (Kennedy and Stubbs, 2006; Trivedi et al., 2016).

4.2. Changes in bacterial communities in the bulk and rhizosphere soils of rice

Plants shape their associated microbial communities to acquire a microbiome that can rapidly adapt to biotic and abiotic stresses. This activity is of special importance in Doñana paddies, where rice plants are selected for their high production and salt stress resistance; this selection may have decreased their ability to interact with soil microorganisms, similar to other modern plant varieties (Pérez-Jaramillo et al., 2016). We analysed the bacterial communities in the bulk and rhizosphere soils of rice during the crop season, which was previously performed in similar complex systems with extensive fertiliser use (Ho et al., 2017).

Table 3

Main topological properties of bacterial co-occurrence networks calculated for bulk soil and rhizosphere samples. Values in brackets represent the total number of modules calculated for each network. Asterisks indicate significant differences among type of soil (Student's t-test). Confidence level of 95 % were selected in all cases.
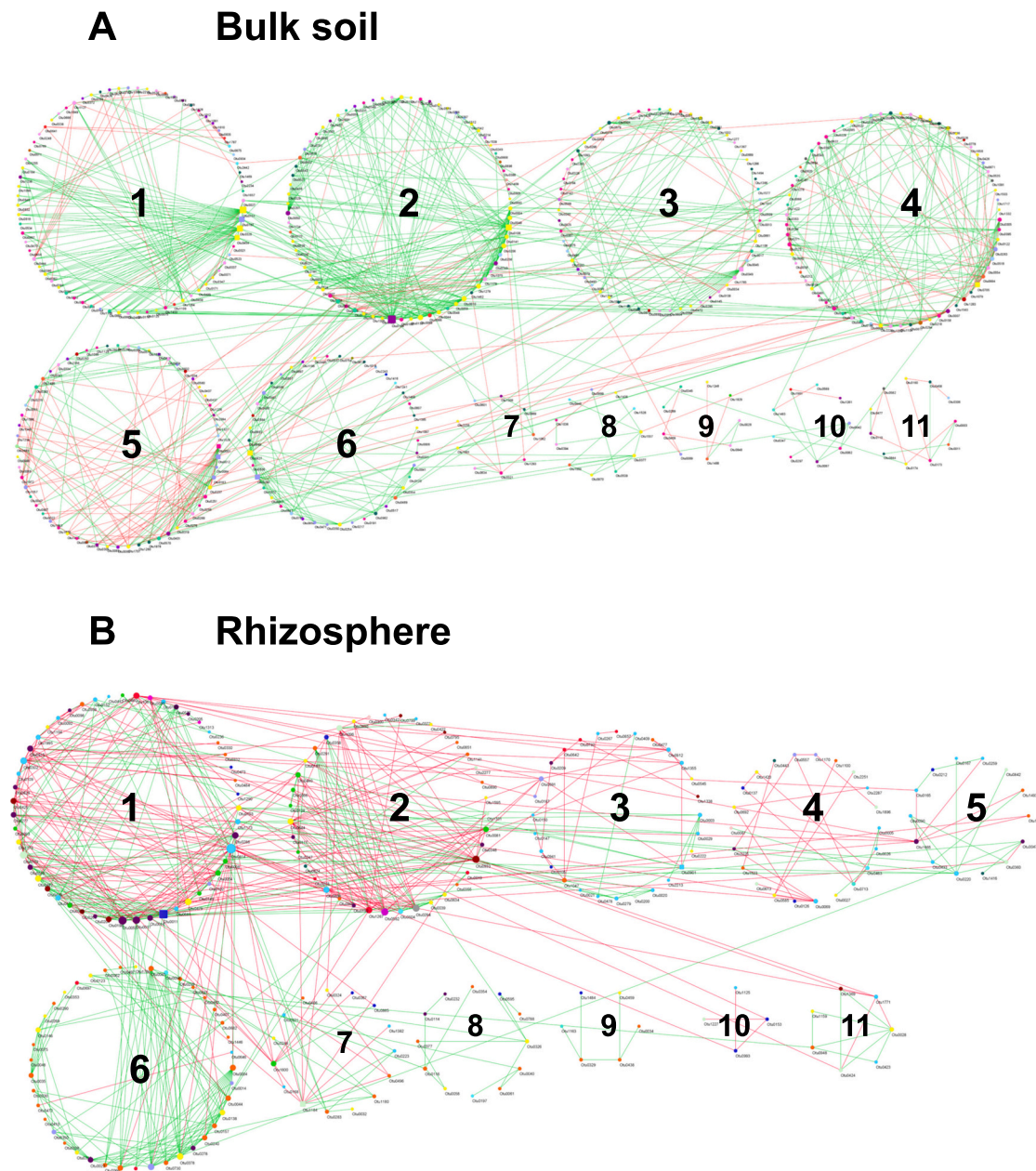
	Similarity threshold (St)	R^2 of power-law	Total nodes	Total links	Positive edges (PEP)	Average degree (avgK)	Average clustering coefficient (avgCC)	Geodesic distance (GD)	Modularity (M)
Bulk soil	0.880	0.894	591	1063	75.45 %	3.597	0.132*	5.582*	0.744* (74)
Rhizosphere	0.850	0.911	279	624	54.81 %	4.473	0.125*	4.546*	0.567* (28)

Fig. 3. Co-occurrence networks of total bacterial communities in bulk soil (A) and rhizosphere (B). Green and red lines connecting nodes indicate positive and negative interactions, respectively. Colour of nodes represent OTUs from different phyla. Square-shaped nodes represent module hubs. Numbers represent the modules.

Our results showed a different bacterial structure in the rhizosphere than in the bulk soil. Richness and diversity indices were generally lower in the rhizosphere than in the bulk soil (Fig. 2). The fact that the rhizosphere microbiota differs from bulk soil microbiota is well documented in other cereal crops, such as wheat (Fan et al., 2017; Illescas et al., 2020) and maize (Peiffer et al., 2013). This result is known as the 'rhizosphere effect', first described by Lorenz Hiltner in 1904. This effect may be attributed to the differences in chemical properties of the root exudates, which are influenced by plant-derived signals and organic compounds released during plant growth (Baudoin et al., 2003). These compounds, which are mainly carbohydrates, carboxylic acids, and amino acids, may be a driving force for bacterial growth, which also occurs in other crops (Mendes et al., 2013; Haichar et al., 2014). In our study, bacterial genera involved in the N and S cycles were highly represented in the rhizosphere. They include different genera of the Rhizobiales order, which fix N_2 in soils, and Desulfocapsaceae family, which

are sulphate-reducing bacteria that perform anaerobic respiration using sulphate (SO_4^{-2}) as a terminal electron acceptor (Galushko and Kuever, 2015). In contrast, two of the most represented genera in paddy soils, *Marmoricola* and *Thiobacillus*, were excluded from the rhizosphere (Fig. 2D). These results indicated that reduced denitrification and S oxidation occurred near the roots. However, how rice roots influence the conformation of the rhizosphere bacteriome is largely unknown. Future studies using other rice varieties will clarify the importance of the interaction between the plant and the soil bacteriome of this and other protected wetlands.

4.3. Bacterial community changes at different plant phenological stages

Plant requirements change during plant growth and development. Thus, the rhizosphere bacterial community structure depends on plant nutritional requirements and phenological stage (Xiong et al., 2021).

This fact was corroborated in our study, as we found distinct bacterial communities in the rhizosphere soil during the vegetative, reproductive, and ripening stages (Table 2). During the vegetative stage, cyanobacteria, including the genera, *Nodosilinea* PCC7104, *Phormidium* MBIC10003, and an unclassified genus belonging to the family Leptolyngbyaceae, were detected in the rhizosphere. Cyanobacteria are photosynthetic prokaryotes commonly found in rice soils (Prasanna et al., 2009; Iniesta-Pallarés et al., 2021). They are used as plant biofertilizers because they provide phytohormones (auxins, gibberellins, and cytokinins), amino acids, polysaccharides, and siderophores (Singh et al., 2016). The high abundance (20.10 ± 12.17 %) of *Nodosilinea* PCC7104, in the rhizosphere during the vegetative stage may indicate specific plant nutritional requirements. We also detected a high proportion of *Pseudomonas*, a genus that includes pathogenic strains but also several plant-beneficial ones (Passera et al., 2019). Beneficial *Pseudomonas* strains activate defence responses in plants and promote plant growth through P and Fe solubilisation and phytohormone production, among other mechanisms (Sah et al., 2021). These two rhizosphere bacterial genera, specifically during the vegetative stage, may be indicative of a specific plant dependence on beneficial bacterial compounds.

The reproductive stage, which comprises panicle initiation, is an important stage as it has major effects on pollen viability and grain yield (Khatun and Flowers, 1995; Abdullah et al., 2002). During the reproductive stage, we found significantly increased proportions of aerobic bacteria adapted to high salinity aquatic environments, such as unclassified genera from the Mixococcales order and *Halomonas* genus. *Halomonas* is one of the most representative taxa among moderately halophilic bacteria (Arahal and Ventosa, 2006). Recruitment of halotolerant bacteria to the plant rhizosphere may be a response to salt stress. Further studies on paddy soils exposed to different salt stress conditions will reveal the importance of these genera in osmotic stress alleviation in plants.

During the ripening stage, the rhizosphere had increased numbers of anoxic bacteria, such as *Pleomorphomonas*, an N_2-fixing bacterium that has been previously isolated from *Oryza sativa* (Xie and Yokota, 2005), and other anoxic bacteria, including unclassified genera from the Desulfovibrionaceae family, which are sulphate-reducing, among other opportunistic bacteria (Table 2). In the rhizosphere, finding aerobic bacteria in the early stages of plant development and anaerobic bacteria in mature plants may be indicative of the age and function of rice roots; for example, flooded paddy soils are typically anaerobic; however, young rice plants have well-developed aerenchyma, which favour oxygen diffusion (Schmidt et al., 2011).

4.4. Co-occurrence networks in the bulk and rhizosphere soils of rice

Co-occurrence networks are powerful tools complementary to analysing microbial diversity, offering associative-level information on multiple types of microorganisms (Barberán et al., 2012; Tao et al., 2018). Our co-occurrence network analyses revealed that, in contrast to the bulk soil, the rhizosphere soil contained a lower number of nodes, many of which showed negative interactions. These negative interactions may reflect the strong influence of biotic and abiotic factors, leading to bacterial adaptation strategies to cope with new situations (Xie et al., 2022). These factors may include excess fertiliser, osmotic stress by salinity, or other abiotic factors (Asiloglu et al., 2021). Importantly, only two OTUs were identified as network hubs in the rhizosphere soil, which were ascribed to the phyla, Proteobacteria and Cyanobacteria. Network hubs are key species transmitting information from the environment to the interconnected microbial network (Van der Heijden and Hartmann, 2016). Our findings indicate that such highly interconnected species benefit the entire system and may benefit plant health, as they mediate between the plant and its microbiome.

The bulk soil bacterial networks showed a significant increase in modularity, GD, and avgCC values compared to that of the rhizosphere.

This result usually reflects a strategy of 'protection' by the bacterial community against possible abiotic and biotic stresses, thereby preparing this community against changes and preventing disturbances from spreading throughout the network (Munir et al., 2022). In bulk soil, bacterial keystones were the most abundant bacteria in the soil, such as those from the SBR1031 order and Anaerolineae class. Members of the Anaerolineae class have previously been identified as keystone species in rice soils, and were critical in the microbial networks during the rice stage of crop rotation (Xie et al., 2022). Taxa belonging to the Anaerolineaceae family can synthesise adherence proteins that promote cellular attachment and facilitate cell aggregation and biological interactions (Xia et al., 2016). Their metabolic activity has positive effects on hydrogenotrophic methanogens, providing acetate, CO_2, and H_2 from sugars derived from rice plants (Sheng et al., 2013; Xia et al., 2016). These features confirmed the role of these species as keystone bacteria under real ecological circumstances.

5. Conclusions

In this study, we assessed the microbial communities in a rice field in southwestern Spain in bulk and rhizosphere soils. These soil compartments had a high impact on the bacterial community structure. Specific taxa were found in the rhizosphere soil, indicating a pressure selection of microorganisms that might influence the nutritional requirements of the plant. The structure of these bacterial communities changes with progressing plant growth, which explains their resistance to abiotic stresses, such as high salinity. Analysis of co-occurrence networks in the rhizosphere revealed many negative connections between community members, that may be caused by biotic and abiotic stresses. In conclusion, analysing microbiological community structures can be used to assess the impact of intensive rice cultivation.

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Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

Data availability

Data will be made available on request.

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