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Research article

How binomial (traditional rainfed olive grove-*Crocus sativus*) crops impact the soil bacterial community and enhance microbial capacities

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ABSTRACT

Intercropping can favour the yield of the main crop. However, because of the potential competition among woody crops, this system is rarely used by farmers. To increase knowledge about the intercropping system, we have explored three different combinations of alley cropping in rainfed olive groves compared to conventional management (CP): (i) *Crocus sativus* (D-S); (ii) *Vicia sativa/Avena sativa* in annual rotation (D-O); and (iii) *Lavandula x intermedia* (D-L). Different soil chemical properties were analyzed to evaluate the effects of alley cropping, while 16S rRNA amplification and enzymatic activities were determined to study the changes that occurred in soil microbial communities and activity. In addition, the influence of intercropping on the potential functionality of the soil microbial community was measured.

Data revealed that the intercropping systems highly affected the microbial community and soil properties. The D-S cropping system increased soil total organic carbon and total nitrogen that were correlated with the bacterial community, indicating that both parameters were the main drivers shaping the structure of the bacterial community. The D-S soil cropping system had significantly higher relative abundances of the phyla Bacteroidetes, Proteobacteria, and Patescibacteria compared to the other systems and the genera *Adhaeribacter*, *Arthrobacter*, *Rubellimicrobium*, and *Ramlibacter*, related to C and N functions. D-S soil was also related to the highest relative abundances of *Pseudoarthrobacter* and *Haliangium*, associated with the plant growth–promoting effect, antifungal activity, and a potential P solubilizer. A potentially increase of C fixation and N fixation in soils was also observed in the D-S cropping system. These positive changes were related to the cessation of tillage and the development of a spontaneous cover crop, which increased soil protection. Thus, management practices that contribute to increasing soil cover should be encouraged to improve soil functionality.

1. Introduction

The traditional rainfed olive grove is nowadays fundamental from the point of view of economic and territorial compatibility as set out in FAO Statistics, Food and Agriculture (FAOSTAT, 2020). It can generate employment (it employs more than 1,000,000 people in the Mediterranean area per year) and be the economic mainstay of large areas of the Mediterranean Basin, being one of the sectors responsible for curbing rural depopulation (de Sosa et al., 2021). Moreover, the climatic requirements linked to this crop mean that up to 98% of the world's cultivated olive area is located in the Mediterranean Basin according to the Retrieved from Food and Agriculture (FAOSTAT, 2017; Youness et al., 2022). However, the conventional tillage (CT) carried out on olive grove soils leads to soil degradation, causing soil organic carbon (SOC) and Nitrogen (N) losses by the rapid mineralisation of soil organic matter (SOM) and high rates of soil erosion, with an associated

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reduction in soil water availability (Rahman et al., 2021; Bouhia et al., 2022). Over the years, different studies have identified agricultural intensification and CT among the main factors causing the high degradation of farming soils, leading to a decrease in biodiversity and soil health and an increase in greenhouse gas (GHG) emissions (Bonou-zin et al., 2019; González-Rosado et al., 2021; Dewi et al., 2022). For this reason, the concept of sustainable agriculture is becoming increasingly relevant today. Management such as zero or minimum tillage with crop residue retention and the use of crop rotations or intercropping means an improvement in soil health by increasing productivity and SOC reserves, thus offsetting GHG emissions and contributing to climate change mitigation and the achievement of sustainable development goals (Corbeels et al., 2019; Bouhia et al., 2021; Aguilera-Huertas et al., 2022). However, while the European Commission, in the Technical Manual of Recommended Management Practices for Recarbonizing Global Soilspromotes the use of such specific management practices (minimum tillage, mulching with pruning residues, cover crops, alley cropping, etc.) as sustainable land management for rainfed woody crops (FAOSTAT, 2021), farmers remain unconvinced of their application. This is because they are concerned about the possible water and nutrient competition between alley or cover crops with the cash crop (tree). This is because in the Mediterranean region, torrential rainfall events alternate with arid periods, with intensive annual droughts (Parra et al., 2022). Nonetheless, when properly selected and managed, alley cropping in olive groves could improve soil health, increasing its fertility, water retention, and productive capacity (Abbas et al., 2020).

Focusing on intercropping (the growth of two or more crops simultaneously in the same area), González-Rosado et al. (2022) proposed this type of management in woody crops by implementing alley cropping as it improves the efficiency of the resources and nutrients used. In particular, the potential of this type of management in woody crops lies in its ability to restore SOC losses derived from unsustainable management (Vicente-Vicente et al., 2016; Bouhia et al., 2023). Authors such as Almagro and Martínez-Mena (2014) showed that SOC losses could be compensated after 20 years of intercropping as green manure. Therefore, in the long term, it is an effective tool to accurately evaluate SOC increases in agricultural soils. In addition, Almagro et al. (2017) showed that the annual amounts of SOC entering the soil as a result of crop diversification exceed carbon (C) losses caused by CO_2 emissions. Another benefit provided by this type of crop is an improvement in soil biodiversity and an increase in the abundance of beneficial soil microorganisms, mostly related to increases in SOC and N, along with the reduction of tillage (Özbolat et al., 2023).

Interest in the soil microbial community has increased in the scientific community given the large contribution of microorganisms to soil functionality and the delivery of ecosystem services through their participation in biogeochemical cycles (i.e. C, N, P, or S cycles). In addition, they present many beneficial effects for transforming and increasing soil nutrients (Urbanová et al., 2015) and for inhibiting soil-borne diseases through active interactions of disease-suppressive microbes under complex and changing environmental conditions (Niu et al., 2020). Therefore, microorganisms have a significant role in soil conservation, plant growth, and crop protection (Baldi, 2021; Tripathi and Gaur, 2021). Intercropping systems have a substantial impact on the composition of microbial communities. This impact is caused by the effect of roots of different plant species interacting with one another and affecting soil microbial diversity, structure, and activity (Lian et al., 2019). In addition, this variation in soil microbial community composition could affect carbon (C) and N dynamics via the intercropping effect (Sun et al., 2009) given the ability of microbial communities to regulate C and N use efficiency (Mooshammer et al., 2014). In this line, Cuartero et al. (2022a) demonstrated the influence of this type of management on the soil bacterial community in melon–cowpea intercropping, which could have a beneficial impact on crops by promoting the growth of beneficial microorganisms such as *Pseudomonas*. In agreement, Koudahe et al. (2022) highlighted the increase in enzymes, nutrients, and soil microbe abundances caused by the roots, which are essential to improve the microecological soil environment and increasing crop yields. Therefore, changes in the soil microbial community caused by intercropping should be considered as these characteristics are essential for maintaining soil health and productivity (Wick et al., 2017; Singh and Kumar, 2021). Previous research has demonstrated this, highlighting that changes in soil microbial diversity and structure are related to plant species, soil physicochemical characteristics, and land use (Li and Wu, 2018). In addition, selecting the type of intercropping is essential to reduce the imbalance among different crops, avoid competition among them, improve facilitation processes, improve soil structure and fertility, and enhance C sequestration and storage (Beillouin et al., 2021). This is why the choice of *Crocus sativus*, *Vicia sativa/Avena sativa* in annual rotation and *Lavandula x intermedia* as diversification crops was motivated by the multiple benefits they generate. From an economic point of view, the cultivation of saffron can be a source of income for the farmer during its life cycle given the yield of its flower and corms, also generating more employment for its maintenance (Khorramdel et al., 2022). From an environmental point of view, planting oats and vetch in rotation and lavender can improve both plant and soil properties. These types of legumes (oats and vetch) are able to fix in the soil not only CO_2 but also N_2 that is incorporated into the soil through root exudates and plant residues, improving its N content (Culumber et al., 2019; Özbolat et al., 2023). In addition, lavandin causes an increase in pollinators, increases biodiversity, and can produce a greater amount of essential oil given the effect of these pollinators, which can be marketed, also increasing the farmer's income (Radev, 2020, 2023). In this way, a more sustainable olive grove could be achieved, maintaining agricultural landscapes and the quality of their soils.

However, the influence and changes in the soil microbial community in intercropped olive groves are not properly understood yet. Therefore, we hypothesise that intercropping and reduced tillage will lead to an improvement in soil chemical properties, microbial activity, and bacterial functionality related to different biogeochemical cycles compared to olive monocultures with bare soil. Hence, this study aimed to (i) evaluate in the short term the impact of minimum tillage and implementation of three alley crops (*Crocus sativus*, *Vicia sativa/Avena sativa* in annual rotation, and *Lavandula x intermedia*) in traditional Mediterranean rainfed olive groves on soil chemical properties, (ii) assess how this strategy can affect soil extracellular enzymatic activities and the composition, diversity, and functionality of the bacterial community related to N, P, and C cycles, and (iii) elucidate the links established between bacterial community and activity and soil chemical properties.

2. Materials and methods

2.1. Study area and experimental design

The study area was established on an experimental centenary rainfed olive grove (*Olea europea* var. *Picual*) in Torredelcampo (Jaen, Andalusia, Spain) (37°50′20″N–3°52′32″W) with two to three trunks under monocropping conditions [conventional tillage (CT)] and herbicide application to keep the soil bare, thus avoiding competition with trees for water) and large growing frames (12 m × 12 m pattern) (Fig. 1). This is the most common olive farm typology in the region. This study was performed for three years (2018/2019–2020/2021). Four experimental plots formed by three rows of olive trees and an area of 2000 m^2 were considered. Briefly, a CT plot (CP) and three different diversified plots were installed in the 2018/2019 growing season. The choice of diversification crops and their planting density was co-defined by farmers and stakeholders in co-decision seminars organised to set up the experimental design in the framework of the H2020 Diverfarming project. Researchers, farmers, and stakeholders co-selected the most suitable crops to favourably coexist with the main crop, able to withstand the climatic conditions of the area and provide environmental and economic

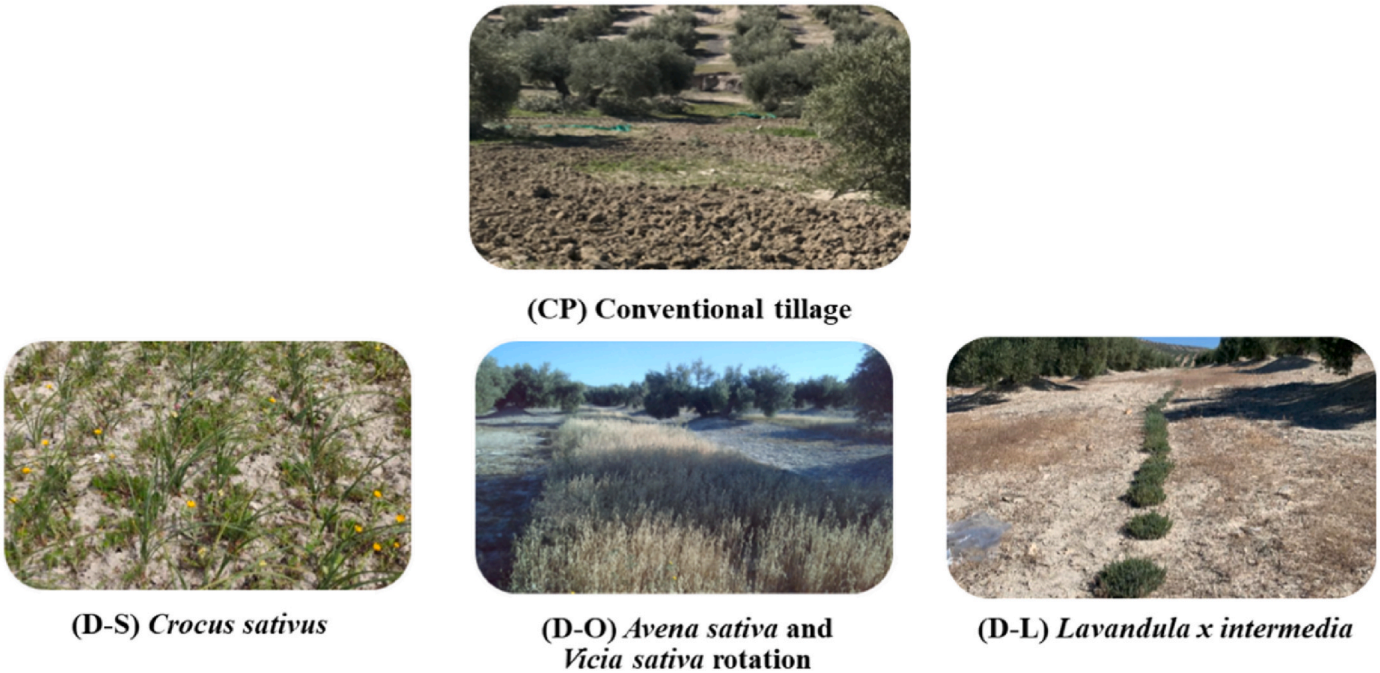

Fig. 1. Different cropping systems and implementation of alley cropping in the study area. (CP) Conventional tillage, (D–S) Olive intercropping with *Crocus sativus*, (D–O) Olive intercropping with *Avena sativa* and *Vicia sativa* in rotation, (D–L) Olive intercropping with *Lavandula* x intermedia.

benefits, with no possible negative effects on the cash crop. The crop diversifications chosen were as follows: an olive grove diversified with *Crocus sativus* (saffron) (D-S), sown once in October 2018 with a seed rate of 2000 kg ha^{-1}; (ii) *Vicia sativa* (vetch) and *Avena sativa* (oats) in annual rotation (D-O) (vetch in 2018/2019 and 2020/2021, oat in 2019/2020) with an annual crop grown in January, after olive harvesting, with a seed rate of 120 kg ha^{-1} and 140 kg ha^{-1}, respectively; and (iii) *Lavandula x intermedia* (lavandin) (D-L), with a plant rate of 12,000 plants ha^{-1}, which required replanting in the second year after the failure of the first time because of adverse weather conditions (Fig. 1). These diversifications were established in the middle of the olive grove alleys, in the cases of oat, vetch, and saffron at a distance of 4.5 m from the olive tree trunk. However, in the case of lavandin, the distance to the trunk was higher (5.5 m) because lavandin is a perennial diversification, and the passage of machinery during olive harvesting had to be allowed. In the CP, the soil was tilled (25 cm) with a cultivator in spring, followed by tine and disc harrowing in the summer. Fertiliser was applied (100 kg ha^{-1} urea, N richness 46%) in February in alternate years after the olives had been harvested. In addition, the pruning residues were incorporated on the soil surface every two years after olive pruning with a shredder, and fungicides and herbicides were applied. Diversification plots (D-S, D-O, and D-L) were established without irrigation, and no herbicides or fertilisers were used. The cultivation work consisted of minimum tillage with a flexible tine harrow (10 cm) for the planting bed of each cover crop (in D-O, tillage was performed annually), mechanical weeding carried out using a manually operated star cono weeder, and the incorporation of olive pruning residues on the soil surface.

A randomised block design with three replicates and five random sampling points was selected for each experimental plot (D-S, D-O, D-L, and CP) ($3 \times 5 \times 4 = 60$ samples). Soil samples were taken in the olive tree alley at 0–10 cm depth in April 2021. The soil samples were separated into two aliquots in the field, one kept at ambient temperature for chemical analyses and the other stored in a cool box with ice for biological studies. The samples were taken immediately to the lab, and soil was sieved <2 mm. The soil for biological properties was stored at −20 °C.

2.2. Chemical soil parameters

The soils of the study area are classified as calcareous Cambisols with some vertic characteristics according to the IUSS WRB Working Group (FAO-ISRIC-ISSS, 2015). From a physicochemical point of view, these soils are characterised by low gravel content, clay texture, basic pH, and low SOM and SOC content, which influences their structure, stability, and quality. They also present low fertility, poor physical conditions, and marginal capacity for agricultural use (González-Rosado et al., 2021; Aguilera-Huertas et al., 2022). It should be noted that this type of soil was formed from the parent rock (limestone) and that their calcareous properties condition their processes and evolution. Total nitrogen (TN) and total organic carbon (TOC) were determined with the elemental C and N analyser CN 802 (VELP Scientifica, Usmate, Italy) (Álvaro-Fuentes et al., 2019). The analysis of soil pH, electrical conductivity (EC), available P, exchangeable Mg, Na, and Ca, $CaCO_3$, NH_4^+, NO_3^-, and particulate organic carbon (POC) contents were performed based on the methods recorded in the *Handbook of Plant and Soil Analysis for Agricultural Systems* (Álvaro-Fuentes et al., 2019).

2.3. Enzymatic activities

The enzyme activities β-1,4-glucosidase (BG), leucine aminopeptidase (LA), β-1,4-N-acetylglucosaminidase (NG), and phosphatase (AP) were measured using the fluorogenic approach according to Marx et al. (2001), and dehydrogenase activity (DH) was measured via a colorimetric procedure according to Von Mersi and Schinner (1991). The detailed protocol for measuring the enzyme activities is recorded in the *Handbook of Plant and Soil Analysis for Agricultural Systems* (Álvaro-Fuentes et al., 2019).

2.4. Soil DNA extraction, PCR amplification, and sequencing

Soil DNA extraction and next-generation sequencing of bacterial 16S hypervariable regions were performed according to Cuartero et al. (2022a). Briefly, soil DNA was extracted from 1 g of soil (wet weight) using DNeasy Power Soil Kit (Qiagen). The quantity and quality of DNA were tested through the Qubit 3.0 fluorometer (Invitrogen, Thermo

Fisher Scientific, USA) and NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). The Ion Torrent™ Personal Genome Machine™ (PGM) was employed to amplify different 16S hypervariable regions using the Ion Xpress™ Plus Fragment Library Kit combined with the Ion Xpress™ Barcode adapter (Thermo Fisher Scientific). Bacterial 16S regions were amplified using an Ion 16 S™ MetagenomicsKit (Thermo Fisher Scientific) with two different degenerate primer sets to amplify regions V2–8 and V3–6, V7–9.

2.5. Sequencing data processing

For raw sequences, barcodes, adaptors, and primers were trimmed by the BaseCaller application. First, the raw sequences were denoised with ACACIA (Bragg et al., 2012) and imported to Quantitative Insights into Microbial Ecology 2 (QIIME2) v2019.1.0 (Bolyen et al., 2019). Then the imported sequences were denoised using the DADA2 algorithm, with the sequences truncated with a $Q > 25$ (Callahan et al., 2016) on average. The sequences were clustered into operational taxonomic units (OTUs) based on 97% similarity with the SILVA reference database using VSEARCH (Rognes et al., 2016). Low-confident OTUs were removed, and the sequences were classified using the VSEARCH consensus taxonomy classifier with the SILVA 132 database. Functional analysis of the bacterial community was done using the PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) algorithm (Douglas et al., 2020). Some predicted functional genes related to N, C, and P cycling in the soil were also studied. The raw sequences were uploaded to the European Nucleotide Archive (ENA) with the study accession code PRJEB58131.

2.6. Statistical analysis

All statistical tests and graphs have been performed using R language v3.6 (R Core Team, 2021). First, normality and homogeneity of variance assumptions were assayed by the Shapiro–Wilk and Levene tests using the car v3.1-0 package (Fox et al., 2007). When the assumptions were met, one-way ANOVA was applied, followed by Tukey's honest post hoc test; as an alternative to parametrical analysis, Kruskal–Wallis test was performed, followed by Dunn's test with Benjamini–Hochberg correction using the FSA v0.9.3 package (Ogle and Ogle, 2017). The differences between the diversified and control systems were shown through the log2 fold change, following this equation: $\text{Log2FC} = \text{Log2(CT)} - \text{Log2(Div)}$, where (CT) corresponds to the control system and (Div) to the diversified system. Principal coordinates analysis (PCoA) based on the Bray–Curtis distance was performed. To test the differences between the diversifications and conventional systems, a permutational multivariate analysis of variance (PERMANOVA) was conducted using the 'betadisper' and 'adonis' functions with 999 permutations from the vegan v2.5-7 package (Oksanen et al., 2020), followed by the 'pairwise. adonis' function with Benjamini–Hochberg corrections for multiple comparisons. The Shannon and Richness indices were calculated using the vegan v2.5-7 package to study the wasagement's effects in alpha-diversity. To evaluate the impact of soil properties and biological properties in the bacterial community, a redundance analysis (RDA) was performed using the vegan package. To evaluate the differential abundance among managements, an analysis of compositions of microbiomes with bias correction (ANCOM-BC) (Lin and Peddada, 2020) was performed in taxonomic classification. All the graphs were performed using the ggplot2 v3.3.6 package (Wickham and Wickham, 2016) grid v3.6.3 (R Core Team, 2020) and ggpubr v0.4.0 package (Kassambra, 2020).

3. Results

3.1. Diversification success and spontaneous cover development

The development of diversifications was not as successful as expected because of the weather conditions (drought), together with the presence of rabbits. However, the change in the soil management to minimum tillage in conjunction with the absence of herbicides allowed the growth of a spontaneous plant cover that accompanied diversifications and greatly influenced the evolution of the different chemical and microbiological properties of the soil. This cover developed to a greater extent in D-S and in D-O, while its growth was lower but also significant in D-L (Table 1). During the first season (2018/2019), the success of the diversification could have been higher given the severe drought conditions, especially in 2019. The water supply needed to be increased as well for the sufficient development of the spontaneous canopy. The cover was less than 20% in D-O and D-L (Table 1); even the lavandin plants dried out and had to be replanted in the following season. The exception was D-S because saffron was planted in October 2018, and at the end of the year, there were sufficient rainfall episodes to allow the nascence of plants. The second season (2019/2020) offered better weather conditions; the cover percentage was higher in D-S and D-O. The cover rate in D-O and D-L was lower than in D-S because of the soil tillage works in January to sow oat seeds and replant lavandin by removing the existing canopy. In addition, the percentage of the alley cover in D-L was lower than in D-S and D-O because lavandin was a perennial cover, and it was necessary to allow the passage of machinery during the olive harvest. Finally, in the third season (2020/2021), the weather conditions were worse (lower rainfall) than in the previous one, and the success of the diversifications decreased, except for D-L, which maintained the same low value of cover caused by the absence of organic residues from the previous harvest, as was the case in D-S and D-O. In addition, in D-L, the low percentage of cover did not slow down soil erosion preventing the improvement of soil conditions (González-Rosado et al., 2021).

3.2. Soil chemical properties

Considering the different soil chemical properties (Table 2), significant differences were found in some of them. Soil EC, Mg, and $CaCO_3$ showed significantly lower values in the diversified soils (D-S, D-O, D-L) than in the CP. The EC values were lower by 21% in D-S, 15% in D-O, and 43% in D-L compared to the CP. Thus, D-L showed the lowest EC. Mg content decreased by 44% in D-S, 40% in D-O, and 45% in D-L compared to the CP. With respect to $CaCO_3$, the decrease was 7% in D-S and D-L and 8% in D-O. However, TOC showed significantly higher content in D-S and D-O, with an increase of 71% and 12%, respectively, compared to the CP (Table 2). In addition, D-S showed higher content of TN (1.78 ± 1.12 g kg^{-1}), NO_3 (50.4 ± 12.0 mg kg^{-1}), POC (1.38 ± 0.56 g kg^{-1}), Na (5.1 ± 2.4 cmol kg^{-1}), and P (7.9 ± 2.0 mg kg^{-1}) compared with the other cropping systems (Table 2), while the highest content of Ca and NH_4^+ was found in D-O, with an increase of 14% for Ca and 11% for NH_4^+ compared to the CP.

Table 1
Percentage of vegetation cover (%) (average ± SD) in Mediterranean olive orchard under different cropping systems over time (2018/19, 2019/20 and 2020/2021 campaigns) (n = 3).

Campaign	Land Management			
	CP (%)	D-S (%)	D-O (%)	D-L (%)
2018/2019	3.8 ± 1.7 aA	63 ± 3.4 aB	19 ± 2.1 aC	8.8 ± 2.7aD
2019/2020	2 ± 1.0bA	88 ± 2.3bB	54 ± 3.1bC	19 ± 2.1bD
2020/2021	8.8 ± 2.1 cA	75.4 ± 2.9 cB	37.6 ± 3.4 cC	20 ± 1.6bD

SD: Standard deviation; Conventional tillage: CP; *Crocus sativus*: D-S; *Avena sativa and Vicia sativa in rotation*: D-O; *Lavandula x intermedia*: D-L; n = Sample size. Numbers followed by different lowercase letters within the same column present significant differences ($p < 0.05$) for the same soil management among the three time periods. Numbers followed by different capital letters within the same row present significant differences ($p < 0.05$) between the different soil management during the same time period.

Table 2
Physicochemical soil properties under different cropping systems.

	CP	D-S	D-O	D-L	p-value
pH	9.06 ± 0.28	9.06 ± 0.18	9.41 ± 0.29	9.12 ± 0.03	0.200
EC (dS m^{-1})	0.14 ± 0.10 ab	0.11 ± 0.12 a	0.12 ± 0.02 a	0.08 ± 0.01 b	0.010
TOC (g kg^{-1})	4.38 ± 0.76 b	7.61 ± 1.78 a	4.79 ± 0.77 b	4.38 ± 0.70 b	0.000
POC (g kg^{-1})	0.71 ± 0.45	1.38 ± 0.56	1.04 ± 0.21	1.02 ± 0.98	0.300
TN (g kg^{-1})	0.80 ± 0.18 ab	1.12 ± 0.24 a	0.83 ± 0.08 b	0.83 ± 0.21 ab	0.046
NH_{4+} (mg kg^{-1})	7.12 ± 1.38 ab	4.20 ± 0.44 b	8.02 ± 1.77 a	5.92 ± 2.67 ab	0.019
NO_3^- (mg kg^{-1})	41.5 ± 5.7	50.4 ± 12.0	47.5 ± 5.8	40.7 ± 11.3	0.200
P (mg kg^{-1})	5.4 ± 3.1	7.9 ± 2.0	3.2 ± 3.6	4.1 ± 3.2	0.100
Ca (cmol kg^{-1})	36.8 ± 8.6	41.3 ± 4.0	42.6 ± 6.0	37.6 ± 7.7	0.480
Mg (cmol kg^{-1})	3.84 ± 1.32 a	2.15 ± 0.27 b	2.29 ± 0.14 b	2.11 ± 0.27 b	0.003
Na (cmol kg^{-1})	1.9 ± 1.2	5.1 ± 2.4	2.7 ± 3.4	3.6 ± 1.4	0.180
$CaCO_3$ (%)	34.50 ± 0.49 a	32.06 ± 0.83 b	31.69 ± 1.57 b	32.08 ± 0.59 b	0.010

n = 5 (mean ± sd); D-S, Olive with *Crocus sativus* (saffron); D-O, Olive with *Vicia sativa* (vetch) and *Avena sativa* (oats) in rotation; D-L, Olive with *Lavandula x intermedia* (lavandin); CP, Olive with conventional tillage. pH, EC, electrical conductivity; TOC, total organic carbon; TN, total nitrogen; NH_4^+ total ammonium; available Ca, Mg, Na and P; NO_3^- total nitrate; POC, particulate organic carbon; $CaCO_3$, calcium carbonate.

3.3. Extracellular enzyme activities

DH was significantly higher ($p < 0.001$) in intercropped systems D-S and D-O (58% and 49%, respectively, compared to the CP). BG, LA, NG, and AP activity were significantly higher in D-S, with an increase of 200% in AG, 100% in LA, 60% in AP, and 40% in BG compared to the CP. Enzyme activities also significantly increased in D-O by 80% in AG, 73% in LA, 18% in BG, and 5% in AP compared to the CP (Fig. 2) (**Suppl. Table 1**).

3.4. Bacterial diversity and bacterial composition

A total of 1,128,820 reads with 5929 different OTUs were obtained before rarefaction at 35,211 reads per sample. D-S soil showed the highest soil bacterial diversity significantly compared to the D-L treatment (Shannon index) (Fig. 3A). No significant differences were found in bacterial richness among treatments; however, the results showed a trend to increase bacterial richness in D-O and D-S. The soil bacterial community structure significantly shifted because of different diversifications, as shown in the PCoA analysis (Fig. 3B, PERMANOVA F = 0.250; P = 0.001). The study revealed significant differences in bacterial community between non-diversified soils (CP) and all crop diversifications and among the different diversifications (D-S, D-O, and D-L) (Fig. 3B, Suppl. Table 2).

The ten most abundant bacterial phylum were identified (Fig. 4A), and the five most abundant were Actinobacteria (53.55%–37.99%), Proteobacteria (30.53%–24.69%), Gemmatimonadates (11.14%–9.94%), Planctomycetes (4.83%–4.40%), and Acidobacteria (4.98%–3.35%) (Fig. 4A; Suppl. Table 3). D-S soil showed, compared to the CP, a significantly higher relative abundance of Bacteroidetes (74% higher), Proteobacteria (36% higher), and Patescibacteria (1.29% higher) and a significantly lower relative abundance of Acidobacteria (82% lower) and Actinobacteria (76% lower) (Fig. 4A; Suppl. Table 3). Twenty-three genera were identified with relative abundance >0.5%, and only 12 showed significant differences among cropping systems (Fig. 4B, Suppl. Table 4). The genera *Adhaeribacter*, *Arthrobacter*, *Rubellimicrobium*, and *Ramlibacter* were only identified in D-S, while *Craurococcus*, *Flavisolibacter*, *Gemmatirosa*, *Geodermatophilus*, *Rubrobacter*, and *RB41* showed lower relative abundance in D-S compared to the other systems (Fig. 4B; Suppl. Table 4). ANCOM results showed how D-S diversification affected *Haliangium* and *Pseudoarthrobacter* ($p < 0.01$), which significantly increased, while *Rubrobacter* significantly ($p < 0.05$) decreased compared to the CP. On the other hand, D-O promoted the growth of *Pseudoarthrobacter*, with significantly higher ($p < 0.01$) relative abundance than the CP. D-L significantly ($p < 0.05$) contributed to the increase in the relative abundance of *Rubrobacter* and *Sphingomonas*. *Haliangium* showed especially ($p < 0.001$) lower relative abundance in DL than the CP.

3.5. Linkages between bacterial community and extracellular enzyme activities and soil chemical properties

Two different RDAs were performed to study the effect of soil

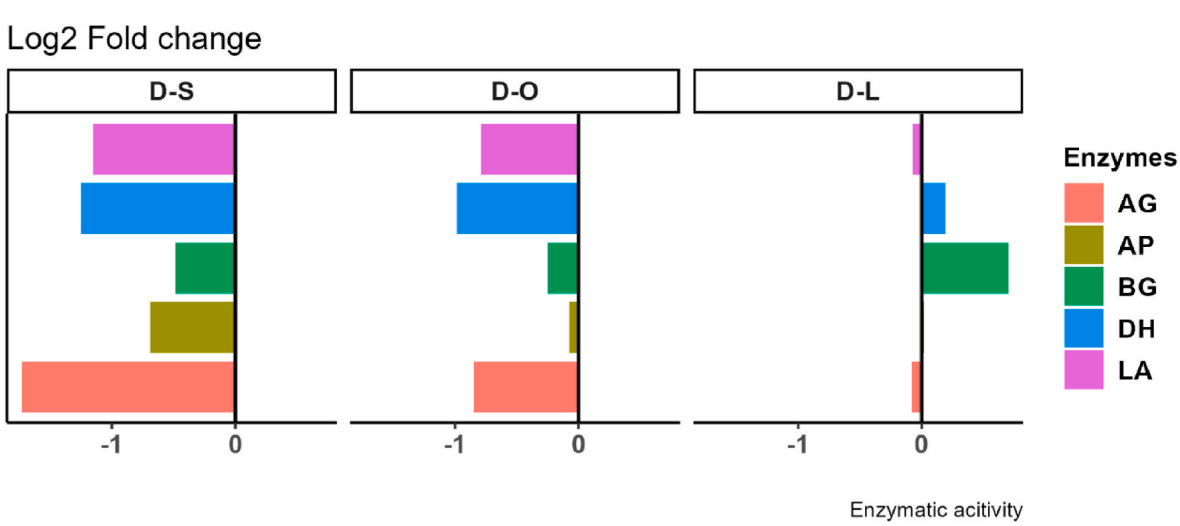

Fig. 2. Log2 fold change of soil extracellular enzymatic activities under different cropping systems. The negative number indicate higher content in diversified cropping system compared with the control. DH, dehydrogenase activity; BG, β-1,4-glucosidase; LA, Leucin aminopeptidase; AP, phosphatase; NG, β-1,4-N-acetylglucosaminidase; D-S, Olive with *Crocus sativus*; D-O, Olive with oat; D-L, Olive with *Lavandula x* intermedia. n = 5 (mean ± sd).

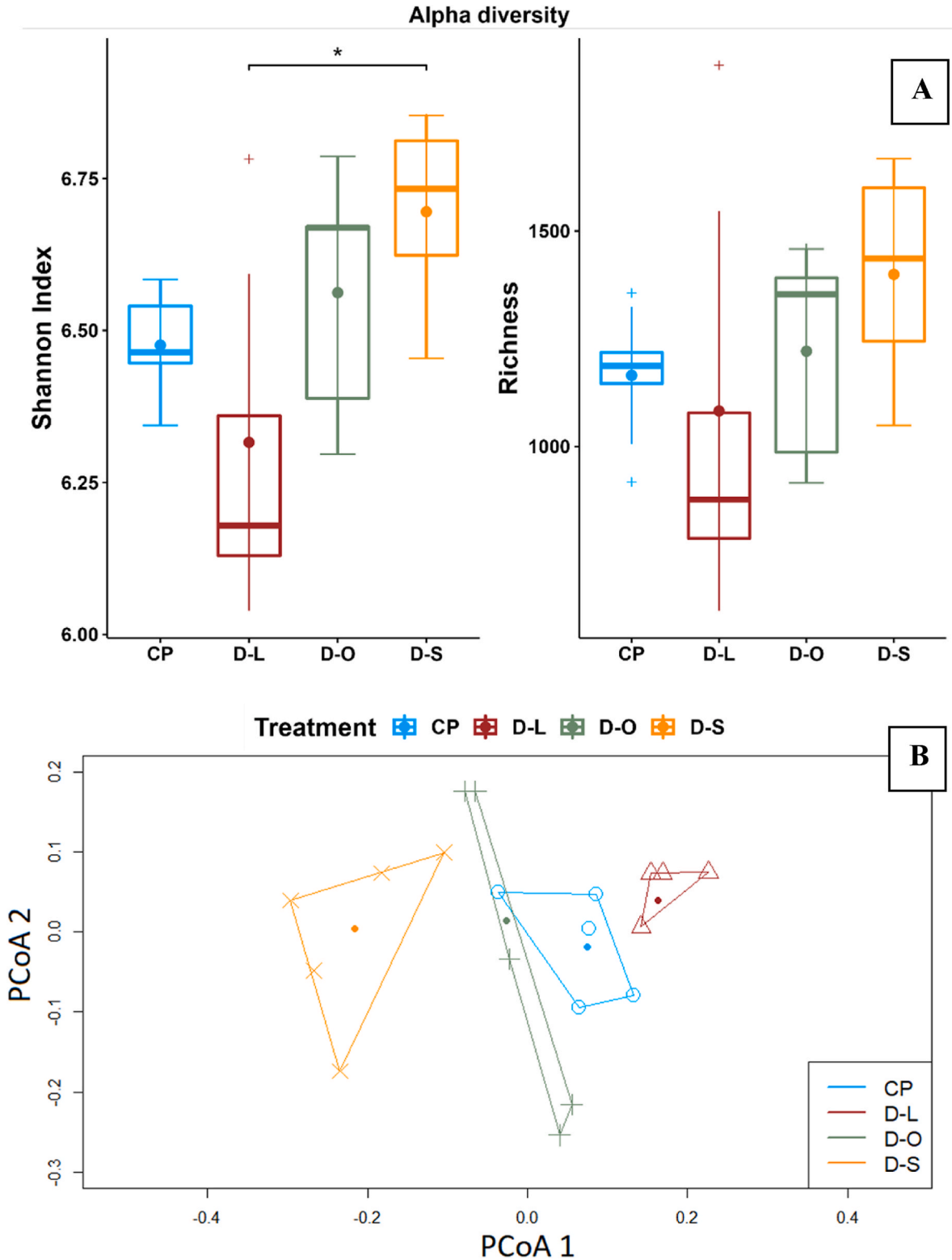

Fig. 3. Alpha diversity and Principal Coordinate Analysis of soil bacterial community based on 16rRNA sequences of different cropping systems. P-value: *, <0.05; **, <0.01; ***, <0.001; D-S, +; outliers, Olive with *Crocus sativus*; D-O, Olive with oat; D-L, Olive with *Lavandula x intermedia*. n = 5 (mean ± sd).

properties and enzymatic activities on the bacterial community (Fig. 5). The impact of soil chemical properties explained 36% of microbial community variance, where the constrained axes RDA1 and RDA2 explained 16.38% of the variation, and showed a significant effect of EC and Mg ($p < 0.05$) (Suppl. Table 5A). The bacterial communities of the diversified soils (D-S, D-O, and D-L) were separated from the CP by Mg, EC, and $CaCO_3$. The bacterial community in D-S was significantly related to higher contents of TOC and TN, while D-O and D-L were related to higher levels of NH_4^+.

Enzymatic activities DH, BG, LA, AP, and AG explained 28% of the total variance of the soil bacterial community (Suppl. Table 5B), where RDA1 and RDA2 contain 14.77% of the variation. The bacterial

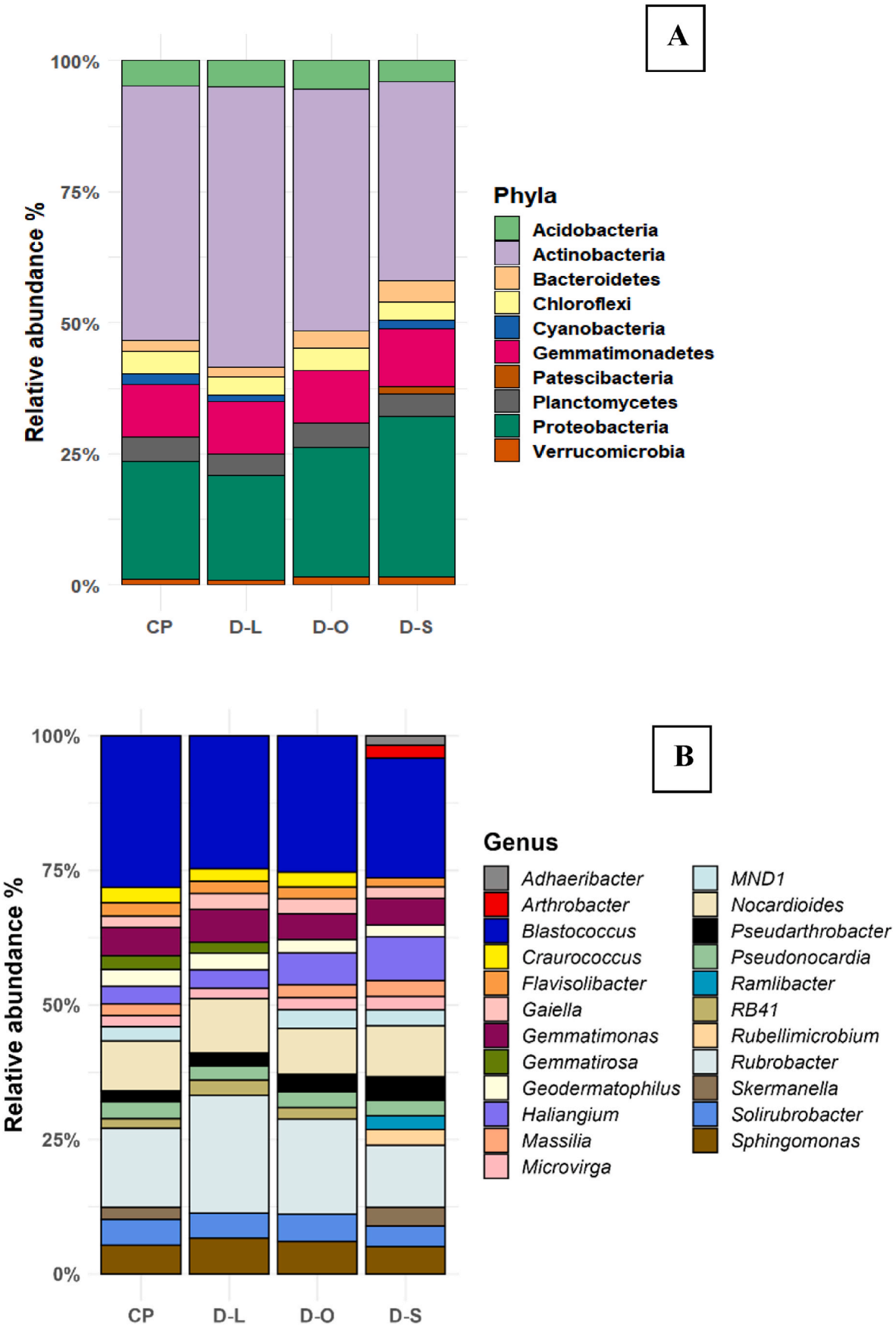

Fig. 4. Relative abundance of soil bacterial phyla (>1%) (A) and genus (>0.5%) under different cropping systems. D-S, Olive with *Crocus sativus*; D-O, Olive with oat; D-L, Olive with *Lavandula x intermedia*. n = 5 (mean ± sd).

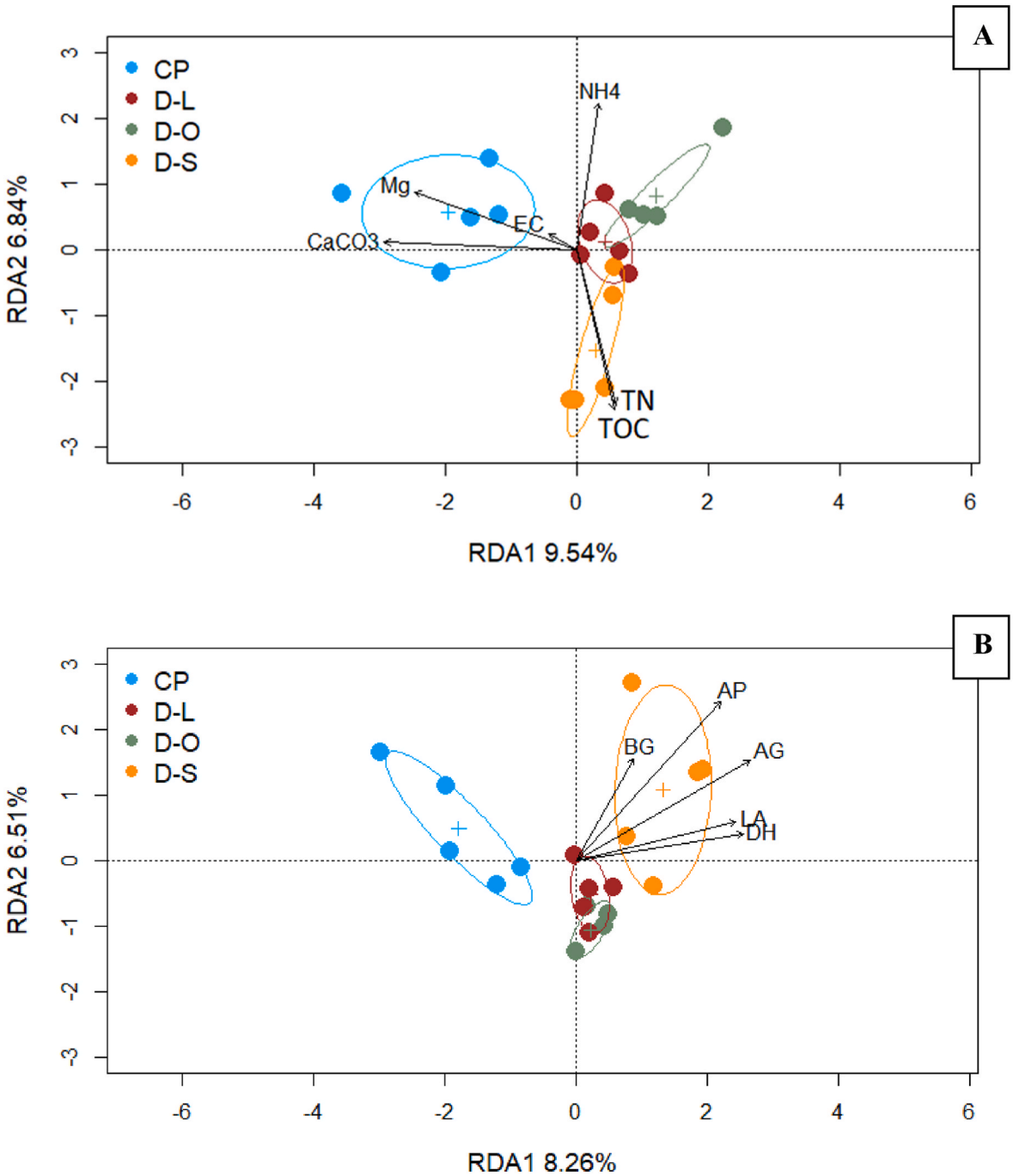

Fig. 5. RDA of (A) soil properties and (B) enzyme activities with microbial community under different cropping systems. D-S, Olive with *Crocus sativus*; D-O, Olive with oat; D-L, Olive with *Lavandula x intermedia*.

communities of the diversified soils (D-S, D-O, and D-L) were separated from the CP (Fig. 5B). The bacterial community of D-S was strongly related to high enzyme activities, and the microbial community of the diversification with *Crocus sativus* (DS) was highly influenced by all the enzymatic activities.

Some abundant microorganisms were significantly positively correlated with soil properties, such as *Blastococcus* with EC ($p < 0.05$), *Gemmatimonas* with Mg ($p < 0.05$), and *Rubrobacter* with $CaCO_3$ ($p < 0.05$). In contrast, there was a negative correlation between *Haliangium* and Mg ($p < 0.05$) and *Haliangium* and *Pseudoarthrobacter* and $CaCO_3$ ($p < 0.001$ and $p < 0.05$, respectively). Regarding enzymatic activities, *Rubrobacter* negatively correlated with DH and LA ($p < 0.05$).

3.6. Potential functionality of the microbial community

3.6.1. C cycling

C degradation genes associated with different substrates' degradation were affected by diversification except for alpha-glucosidase (*malZ*), related to the degradation of cellulose (Suppl. Table 6). The genes that degrade lignin [catalase and glutathione peroxidase (*gpx*)] showed significantly higher values in D-S, while those that degrade hemicellulose (*FUCA*) showed significantly higher values in D-O (Fig. 6; Suppl. Table 6). The gene *ppc* related to C fixation showed higher values in D-S, followed by D-O, although no significant differences were observed between the cropping systems.

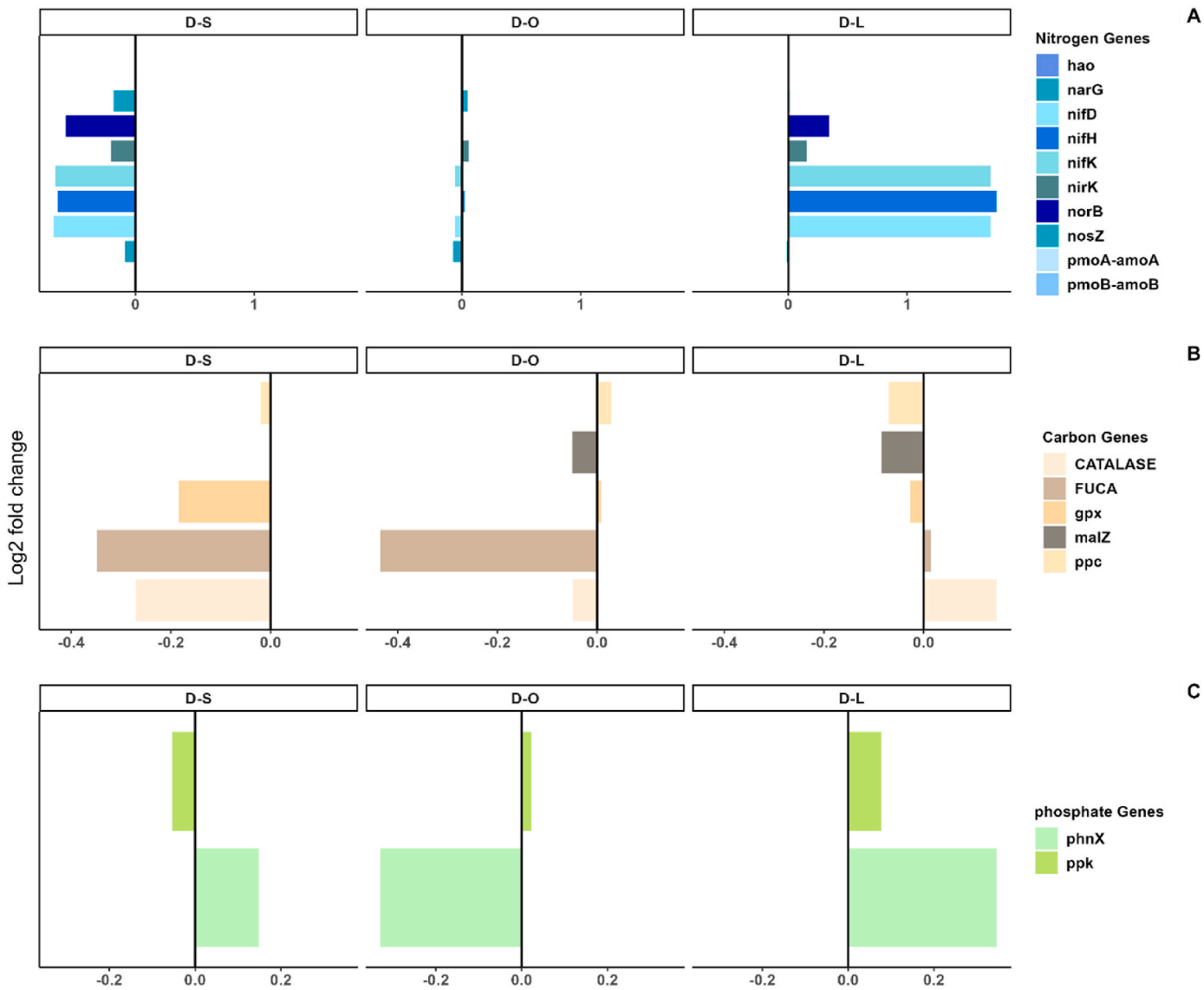

Fig. 6. Barplot showing the log2 fold change of potential (A) nitrogen (B) carbon and (C) phosphate gene expression under different cropping systems. Hao, amoA and amoB were removed. D-S, Olive with *Crocus sativus*; D-O, Olive with oat; D-L, Olive with *Lavandula* x intermedia; CP, Olive with conventional tillage. The negative number indicate higher content in diversification cropping system compared with the conventional tillage.

3.6.2. N cycling

Genes involved in N-fixation (nifD, nifK) and denitrification (*norB*) showed significantly increased abundance in D-S compared to the CP. However, no significant difference was found in genes involved in the nitrification process (*amoA/AmoB)* (Fig. 6; Suppl. Table 6). Interestingly, D-L showed a contrary effect on the nitrogen cycle (*norB*, *nirK*, *nifK*, *nifH*, and *nifD*) than D-S, whereas D-O showed comparable functionality to the CP system (Fig. 6; Suppl. Table 6).

3.6.3. P cycling

Phosphonatase (*phnX*), involved in organic P mineralisation, showed higher abundance in D-O, although no significant differences were observed among the cropping systems. Polyphosphate kinase (ppk), engaged in inorganic P solubilisation, showed significantly higher abundance in D-L (Fig. 6; Suppl. Table 6).

4. Discussion

Several studies have been conducted on cover crops in the alleys of olive groves (de Torres et al., 2021; López-Vicente et al., 2021). However, little is known about the growth of a second crop in these alleys (alley cropping) (González-Rosado et al., 2022). Satisfactory intercropping should increase land-use efficiency and promote positive crop interaction (Hauggaard-Nielsen and Jensen, 2005; Cuartero et al., 2022b), where the interaction should shift the soil microbial community, which produces extracellular enzymes responsible for degrading plant residues and maintaining nutrient cycles (Curtright and Tiemann, 2021), The spontaneous vegetation cover, observed mainly in D-S, improved TOC and POC in soils, owing to the accumulation of litter and root exudates (Namatsheve et al., 2020), along with an increase of soil nutrients. This increase agrees with previous studies (Zheng et al., 2018; Yan et al., 2022) that have shown the beneficial effect of sustainable soil management, such as crop diversification, on C dynamics. According to previous authors, this sustainable strategy has contributed to increasing SOC stocks, confirming C sequestration in agricultural soils, as well as increasing soil nutrients. In addition, other chemical parameters such as EC and $CaCO_3$ decreased when comparing intercropping with the CP. This implies an improvement in the study soils as a result of intercropping by reducing their salinity and carbonate content, providing greater fertility in basic soils such as those studied, since high carbonate content

has repercussions on the low availability of nutrients in the soil (Ball et al., 2020). However, available Mg did not improve in the intercropping plots compared to the CP. This may be due to the extreme deterioration that the soils suffered before the diversifications were implemented as a consequence of the mechanised management carried out on them, combined with the severe environmental conditions of the area (with very high temperatures, the absence of rainfall events, and extreme evapotranspiration rates), which makes it difficult in the short term to evaluate the effect of diversification on these parameters, finding soils poor in SOM and easily erodible (Martínez-Mena et al., 2021; González-Rosado et al., 2022).

Concerning soil enzyme activities, Curtright and Tiemann (2021) observed that the intercropping system significantly increased enzyme activities by an average of 13% and the effect depended on the enzyme category, the crops, and experimental and environmental factors. This effect has also been observed in our experiment, with differences in enzyme activities caused by the increase in soil cover by alley crop (*Crocus sativus*, *Vicia sativa/Avena sativa* or *Lavandula x intermedia*), together with the spontaneous cover developed by the change in soil management (minimum tillage instead of CT). The increase in DH activity in D-S reflects higher microbial activity in the soil diversified with saffron, while the increase in AP activity suggests an increase in P bioavailability that can be absorbed by microorganisms and plants (Huang et al., 2019). Crop diversification also led to a potential increase in the bioavailability of glucose molecules (an important C energy source) for the growth and activity of soil microorganisms (Merino et al., 2016), reflected by increased BG activity. Furthermore, the increase in LA and NG enzyme activities represents the enhanced release of plant-available N from organic compounds and so improved potential soil fertility (Sinsabaugh et al., 2008). The regulation of soil properties and enzyme activities in D-S reflects a significant increase in bacterial diversity attributed to the rise of different plant species aboveground (saffron plus spontaneous cover) with positive root interaction and rhizodeposition, which may have a positive impact on bacterial diversity and structure (Guo et al., 2018; Santonja et al., 2017). The bacterial community in D-S was correlated with TOC and TN, indicating that both parameters are key factors shaping the structure of the community. Similar results were observed by Lian et al. (2018) in sugarcane–soybean intercropping and Wang et al. (2021) in amended soils. According to Ramirez et al. (2010), changes in nutrients also affect microbial communities since increases in available substrates might increase the activity of copiotrophs in soil (Fierer et al., 2012).

The diversified soil with *Crocus Sativus* (D-S) had a significant abundance of the phyla Bacteroidetes, Proteobacteria, and Patescibacteria compared to the other cropping systems. Proteobacteria is a major phylum in soil ecosystems, with crucial roles in mineralising numerous soil nutrients and fixing atmospheric N (Agri et al., 2022; Fallah et al., 2021). Accordingly, we have also observed that D-S showed a higher abundance of genes *nifH/nifK/nifD* compared to the other treatments and so a higher capacity to fix atmospheric N. Ambardar et al. (2014, 2016, 2021) and Farda et al. (2022) also showed that Proteobacteria was the most abundant phylum in the saffron rhizosphere.

Bacteroidetes are described as less common and less significant in agricultural soils (Hartmann and Widmer, 2006), and they thrive because of their ability to secrete diverse arrays of carbohydrate-active enzymes that target the highly varied glycans in soil that carry out detritus decomposition (Larsbrink and McKee, 2020). They are abundant pathogen-suppressing members of the plant microbiome that contribute prominently to rhizosphere phosphorus mobilisation (Lidbury et al., 2021). Patescibacteria, which appear only in D-S, can degrade macromolecular substances such as polysaccharides and glycoproteins (Youssef et al., 2015). They are considered anaerobes often found in groundwater environments, and they contain numerous carbohydrate metabolism genes, especially involving basic and intermediate metabolites such as monosaccharides and pyruvates (Tian et al., 2020). This phylum has shown lower percentage in the cabbage–beetroot and celeriac–leek systems (Trinchera et al., 2022) and in the saffron rhizosphere (Farda et al., 2022). Also, Suproniene et al. (2022) observed the enrichment of this phylum in mineral nitrogen–-treated soil, similar to our results with the highest nitrogen levels in D-S soils compared to the other diversifications and CP soils. These results evidence the ability of the diversification strategy associated with minimum tillage to modulate the bacterial community composition, increasing the relative abundance of the microbial groups generally reduced in disturbed agricultural soil (Wolińska et al., 2017).

The significant decrease of Acidobacteria and Actinobacteria in D-S could be due to the rich nutrient soil. Acidobacteria is a group of oligotrophic bacteria typically found in nutrient-poor and highly acidic soil environments, and they can degrade complex and recalcitrant carbon sources (Fierer et al., 2003). Actinobacteria are mainly involved in fast-degrading low biodegradable organic compounds such as hydrocarbons, lignin, and humus (Trinchera et al., 2022). However, genes involved in lignin degradation and SOM turnover (Melero et al., 2006) [catalase and glutathione peroxidase (gpx)] showed significantly higher values in diversified soil with *Crocus sativus* (D-S). This could indicate that although they are in lower abundance, their potential functionality could be higher.

D-S diversification significantly increased the abundance of *Haliangium* and *Pseudoarthrobacter* with plant growth–promoting effects (Zhou et al., 2018; Ham et al., 2022). Moreover, some species of *Haliangium* are producers of haliangicins, known as antifungal compounds (Cuartero et al., 2021), which could indicate the increase of beneficial microorganisms. Meanwhile, other bacterial groups appeared in response to D-S as *Adhaeribacter*, associated with a diverse potential function such as SOM decomposition (Calleja-Cervantes et al., 2015), *Arthrobacter*, related to the utilisation of aliphatic amino acids and aromatic hydrocarbons as sole sources of carbon (Hagedorn and Holt, 1975), or *Ramlibacter*, involved in the reduction of NO_3^- to NO_2^- (Fu et al., 2019).

As the bacterial community structure of diversified soil (mainly in D-S) changed after three years of implementation of this diversification, the predicted gene function of the soil microbial community related to C, N, and P also changed significantly. The analysis of *pcc* genes related to the C4 cycle, the most important pathway to fix CO_2 from the atmosphere in the terrestrial ecosystem (Jansson et al., 2010), showed that D-S affected the C-fixing microorganism promoting C-fixation. This indicates that autotrophic communities are affected by vegetation types and management (Yu et al., 2020). Also, D-S increased lignin degradation (catalase and GpX) and hemicellulose degradation (FUCA), which could induce potential soil loss C accumulation (Chen et al., 2018). The D-S diversification system improved N fixation, indicating a higher abundance of N_2-fixing bacteria, such as *Pseudarthrobacter* (Actinobacteria), that inhabit both plant tissues (e.g. nodules, roots) and the soil–root rhizosphere. This genus can, consequently, supply significant N amounts for plant growth, providing an ecologically acceptable complement or substitute for mineral N fertilisers (Aasfar et al., 2021). The lower amount of NH_4^+ observed in D-S, followed by D-L, indicated a lower source of energy for the oxidising bacteria ammonium, so there would be fewer copies of the *amoA/amoB* genes, as observed in this experiment, indicating the lower presence of ammonium oxidisers (Wankel et al., 2011) compared to D-O and the CP. These results are supported by the high content of TN and NO_3^- in soils from the C–S system compared to the other systems, which may indicate that N is continually being transformed. Furthermore, this could indicate less loss by lixiviation and higher availability of nitrates to increase the productivity and growth of plants (Ayiti and Babalola, 2022).

The denitrification process was also higher in D-S compared to the other diversifications, where the *Ramlibacter* and *Haliangium* genera, involved in the denitrification process (Zhang et al., 2021; Fu et al., 2019), were associated. The importance of this process as a pathway of N loss in agricultural soils is evident. Denitrification is assumed to equal the difference between known inputs and harvest plus leaching outputs. Also, denitrification is the primary source of atmospheric N_2O, an

important GHG that consumes stratospheric ozone (Shcherbak and Robertson, 2019). In the D-S diversification soil, we can observe the increase of norB, principally involved in the last step of the cycle, the reduction of NO to N_2O. However, this diversification (D-S) showed a high abundance of the *nosZ* gene, which codes for the enzyme N_2O reductase, which then catalyses the enzymatic reduction of N_2O to N_2 (Krauss et al., 2017). This process may then control the levels of gases emitted in these ecosystems. Similar results were observed in melon–cowpea intercropping by Cuartero et al. (2022b).

Phosphorous is also an essential macronutrient that plays a vital role in the growth and sustenance of the plant. The availability of inorganic phosphate (Pi) in the soil is suboptimal given its highly reactive nature. Considering that microorganisms are one of the key players in P cycling for its availability to plants over time (Arif et al., 2017), the abundance of Pi-solubilising genes (*ppk*) in D-L and D-S could indicate a higher capacity for the microbial community to access these Pi forms compared to the other cropping systems. According to Li et al. (2014), this gene was mainly distributed in Proteobacteria and Actinobacteria. However, the higher abundance of gene *phnK* indicated higher availability of organic forms for D-O than the other cropping systems. Interestingly, Wang et al. (2021) revealed how the genus *Haliangium*, which was significantly higher in the system D-S, could be involved in soil P removal. Therefore, further studies about this genus as a potential P-solubilising bacteria should be conducted (Duan et al., 2022).

5. Conclusion

The results of this study highlight the importance of intercropping and reduced tillage to enhance soil C sequestration and fertility and to stimulate beneficial bacterial communities in centenary rainfed olive groves. In this sense, the use of saffron in alley cropping and the associated spontaneous vegetation cover showed the highest increase in soil C content and nutrients compared to the other intercropping systems and monoculture. The increased alley vegetation cover was associated with positive effects on soil enzyme activities and bacterial community composition and potential functionality. D-S improved the presence of beneficial bacteria such as *Haliangium*, associated with plant growth–-promoting effects, antifungal activity, and potential P-solubilisation activity. Also, the potential functionality mainly related to C, N, and P cycling indicated that the D-S system potentially increases C fixation and N fixation in soils and the solubilisation of phosphates. These results can encourage further research on the relationship between alley cropping and soil functionality, how other soil organisms such as fungi, protists, or invertebrates respond to alley cropping and contribute to recover soil functionality, and how the links between soil biodiversity and soil health influence aboveground productivity.

Credit author statement

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Declaration of competing interest

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Data availability

The authors do not have permission to share data.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2023.118572.

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