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Bacterial communities show distinctive spatial diversity patterns in productive truffle orchards amended with peat-based substrate

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Abstract
As truffle cultivation expands, growers empirically develop new agronomic management practices aimed at promoting truffle growth such as "truffle nests", localized peat amendments that are supplemented with truffle spore inoculum. Previous research showed that nests contain lower fungal diversity than the surrounding soil, which could encourage its occupation by pioneer species such as *Tuber melanosporum*. However, truffle nests did not quickly stimulate truffle mycelium growth. We hypothesized that the bacterial community from the soil may be the first to colonize nests and that fungal and bacterial diversity in nests would have an inverse relationship. To test this, we characterized the bacterial community of truffle nests, via 16S rRNA gene amplicon sequencing, in two orchards during the two years after establishing the nests. Unexpectedly, we did not find drastic differences in the bacterial diversity inside nests with respect to the bulk soil or the commercial substrate before being introduced in the field. However, Proteobacteria richness in nests was positively correlated to truffle mycelium abundance, which together with a higher relative abundance of Proteobacteria in nests than in bulk soil, indicates a possible underlying factor for the performance of nests in truffle plantations. Fungal and bacterial richness was positively correlated in nests, countering our hypothesis that bacterial diversity would negatively impact fungal diversity.

Keywords *Tuber melanosporum*, Truffle cultivation, Truffle nests, Bacterial community, 16S amplicon sequencing, Proteobacteria

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Introduction

The European black truffle (*Tuber melanosporum* Vittad.) is an ectomycorrhizal fungus that produces below ground ascocarps that are highly appreciated in cuisine due to their unique aroma. Its high prices and the decline of wild harvests have led to the emergence of truffle cultivation not only within its native distribution range, but also in other regions of the world [1, 2]. Nowadays, most black truffle production comes from cultivated orchards that were established with nursery-inoculated tree seedlings [3]. However, successful truffle cultivation is connected to mating and successful ascocarp development [4, 5]. Truffle yields differ drastically across sites and years, frequently in relation to bioclimate and soil factors [6]. Microbial communities are implicated to be important to truffle production, although it is not well understood exactly how microorganisms influence truffle fruiting or its mycelial growth, which eventually gives rise to the fungal sexual gametangia [7, 8].

As truffle cultivation has expanded, growers have introduced new management practices. One of the recent empirically-developed practices is the incorporation of "truffle nests" into orchard soils, hereafter referred to as nests [3]. Nests are made by establishing holes around the host tree that are typically filled with a pH-adjusted (7.5-8.0) peat-based substrate that has been supplemented with truffle spores (0.1–1 g L^{-1} of dry ascocarp). Growers claim that nests can accelerate and increase truffle production yields in young plantations, which has raised questions regarding the factors enhancing truffle fruiting [9]. Truffle spores can serve as nuclear donors in sexual mating, whereas the physical conditions inside nests are thought to play a role in triggering the ascocarp initiation [10, 11]. However, adding spores into nests does not always increase truffle fruiting and the physical changes induced in soil do not quickly stimulate truffle mycelium growth [10, 12]. This led us to hypothesize that the relationship between black truffle and soil microbial communities may underlie anecdotal effects of increased truffle production observed with nests, usually happening two years after they are established. Previous analyses of the fungal communities in nests revealed lower richness and diversity compared to the surrounding soil [12]. Pioneer species such as *T. melanosporum* could benefit if they are able to colonize and occupy this niche. On the other hand, bacterial communities may influence the colonization of this niche, but they have yet to be characterized in nests.

Bacteria are involved in tripartite interactions with mycorrhizal fungi and plant roots in what is called the mycorrhizosphere, acting in some cases as mycorrhiza helper microorganisms [13]. In truffles, bacteria are hypothesized to play a role in the formation and function of both mycorrhizae and ascocarps [8, 14–16]. At the same time, soil prokaryotic communities are affected by the biochemical modifications resulting from *T. melanosporum* metabolism [17–19]. Rhizospheric bacterial communities can be influenced not only by plant root exudates but also by the cohabiting fungal communities, since they compete for common resources [20, 21]. All this leads to the hypothesis that the richness and diversity of the bacterial community in nests will show a negative relationship with both the abundance of truffle mycelium and the richness of the fungal community. Investigating these ecological relations could help to clarify the relevance of the soil microbiome in the presumed nest effect. Statistical techniques such as network analysis or co-correspondence analysis (CoCA) can help disentangle interactions in complex microbial communities, although always with the limitation that co-variation does not necessarily imply direct association [22].

In this study, we investigated the prokaryotic communities in truffle nests during the two years following their establishment, across two truffle orchards. We assessed the diversity and composition of the prokaryotic community of peat-based substrate annually for two years after establishing truffle nests. We also explored patterns of co-variation of the diversity and composition of the prokaryotic community to the abundance of *T. melanosporum* mycelium, as well as to the diversity and composition of the whole fungal community. We expected bacterial communities in truffle nests to quickly change from their first to their second year in the field, since prokaryotic communities have the ability to quickly disperse with water and respond to environmental changes [23, 24]. As a complement, a few samples from the initial peat-based substrate and the bulk soil of plantations were used as baseline references. This study contributes to the field by characterizing prokaryotic communities in *T. melanosporum* nests, and by providing co-variation patterns with truffle mycelium and with the fungal communities using previous qPCR and high-throughput amplicon sequencing data generated from the same sites [12].

Materials and methods

Experimental design and sampling

For this study, two *T. melanosporum* truffle orchards were selected, both in Teruel province (eastern Spain). One truffle orchard was located in Teruel (1090 m a.s.l., mean annual temperature: 11.6 °C, mean annual rainfall: 440 mm) and the other in Mora de Rubielos (1150 m a.s.l., mean annual temperature: 11.1 °C, mean annual rainfall: 520 mm). The climate for both sites is continental mediterranean and the soils are naturally calcareous. The Teruel site consists of a sandy-clay-loam soil developed on Tertiary conglomerates and clays, whereas the

Mora soils are sandy-loam developed on Tertiary siltstones/sandstones (Table S1).

The Teruel orchard was planted in 1999 with *Quercus ilex* L. subsp. *ballota* Samp. as the truffle host tree, whereas the Mora orchard was planted in 2001 with *Q. ilex* and *Quercus faginea* Lam, although for homogeneity purposes we only sampled *Q. ilex*. Both orchards were planted at a density of 278 trees ha^{-1} (6 × 6 m), with seedlings produced in commercial nurseries. The initial mycorrhizal status of seedlings was verified by public authorities following the INIA-Aragón method [25]. The soil was tilled once a year during the pre-productive stage and the trees were annually pruned from the fifth year. The orchards began to produce ascocarps at age six (Mora) and ten (Teruel). Since then, the Teruel site was no longer tilled, whereas the Mora site continued to be tilled once a year, in spring. Since they began to produce, the orchards were irrigated with a sprinkling system, from April to October.

In April 2016, when Mora and Teruel orchards were 17 and 19 years old respectively, we randomly selected 13 productive truffle trees per orchard and established six nests around each tree. A commercial substrate based on *Sphagnum* peat (Turbatruf ® from Projar, Quart de Poblet, Spain: a black peat - white peat - coir - perlite mix 11–5–3–1 in volume, with pH raised to 7.5) was used. Ripe *T. melanosporum* ascocarps (harvested in Mora plantations) were ground, incorporated in the substrate (0.1 g dry ascocarp per liter of substrate) and then the mixture was thoroughly homogenized. Establishing of the nests involved digging holes about 25 cm deep, filling them with about 1.5 L of substrate and re-covering it with soil. The nests were arranged along a 1.5 m-radius circumference centered on the tree trunk. They were within the *brûlé*, the truffle-producing soil around the host tree usually free of herbaceous vegetation.

In April 2017 and in April 2018 (one and two years after establishing the nests, respectively), one sample of nests was taken from each tree of each orchard. Each sample was composed of two subsamples corresponding to two nests of the same tree, which were then mixed. A soil borer of 10 cm depth and 3 cm diameter was used for the sampling, resulting in subsamples of ca. 70 cm^3. All the sampling material was cleaned and disinfected with diluted ethanol from one sample to the next one.

Not all the experimental trees could be sampled, due to the impossibility of finding unperturbed nests (e.g. animal digging, accidental destruction by machinery passage). In the Teruel site we sampled 12 trees in 2017 (nest samples labelled as N1T) and 13 in 2018 (N2T). In Mora we sampled 10 trees both in 2017 and 2018 (labelled as N1M and N2M correspondingly). This resulted in a total sampling of 45 nests. Apart from the experimental design, in April 2016 we took two samples of the commercial peat-based substrate (one for each orchard) prior to its incorporation into the soil, as a reference of the initial conditions of the substrate (labelled as N0T and N0M). The substrate used in both sites formed part of the same batch and was handled jointly up to the moment it was brought to the plantations. With the same rationale, four bulk soil samples were taken in 2018 within the *brûlés* of the orchards, at 20–30 cm of distance from nests and following the same methodology described for the nest samples, to serve as baseline plantation soil references (soil samples labelled as S2T in Teruel and S2M in Mora site).

DNA extraction, amplification and bioinformatic analysis

All samples were air-dried at room temperature and then homogenized by sieving through a 2 mm mesh. The sieve was washed with soapy water and ethanol between samples. Approximately 250 mg of dried soil per composite sample were used to extract genomic DNA with the MagAttract PowerSoil DNA Kit (Qiagen, Hilden, Germany) on a KingFisher Flex Purification System (Thermo Fisher Scientific, Waltham, MA, USA).

The prokaryotic V4 region of the 16S rRNA gene was amplified with the 515 F-806R primers and each library indexed with a unique barcode using a modified PCR primer frameshift protocol initially developed by Lundberg et al. [26] and later adapted by the Earth Microbiome project, as well as our group with some subsequent modifications [27–30]. Sample libraries were normalized with a SequalPrep normalization plate kit (Thermo Fisher Scientific) and pooled together. The pooled amplicon libraries were then concentrated at approximately 20:1 with Amicon Ultra 0.5-ml 50 kDa filters (EMD Millipore, Darmstadt, Germany), according to previous studies [28–30]. Libraries were then sequenced on a MiSeq Illumina platform using the v3 kit 300PE.

Libraries were demultiplexed with a custom bioinformatic pipeline (available at https://github.com/Gian77/Demultiplex-USEARCH) based on USEARCH [31]. After demultiplexing we adopted the Cecilia analysis pipeline (Benucci GMN, available at https://github.com/Gian77/Cecilia) for the subsequent bioinformatic analysis. The Cecilia pipeline performs the following steps: quality control of raw data with fastqc [32], removal of Phix reads by mapping reads to the Phix genome with bowtie2 [33], elimination of primers and Illumina adapters with Cutadapt [34], filtering of reads based on user-specified expected error thresholds [35], trimming and dereplication of sequences, clustering of sequences into 97% operational taxonomic units (OTUs) using UPARSE [36], and taxonomic assignments of representative sequences of each OTU with CONSTAX v2 [37] using SILVA reference database [38].

The DNA extractions used in this study were part of those previously used to study fungal communities in nests [12], which included quantifying *T. melanosporum* mycelium in all soil samples. We incorporate data of both fungal community profiles and *T. melanosporum* mycelium abundance in the analyses presented here.

Statistical analysis

The OTU tables, representative sequences, sample data and taxonomic classification files were imported into the R statistical environment, version 4.4.3 [39] with the R package *phyloseq* version 1.48.0 [40]. Before starting the analyses, we removed PCR and sequencing contaminants with the R package *decontam* (version 1.24.0) using data from negative control samples that were included in the MiSeq libraries [41]. Non-prokaryotic and unclassified taxa were filtered out. To reduce bias due to uneven per-sample sequencing efforts, OTUs were normalized with a Gaussian model (cumulative sum scaling) with the R package *metagenomeSeq* version 1.46.0 [42].

Observed OTU richness, evenness and Shannon's diversity index were selected as α-diversity metrics and determined using the "plot_richness" function in the *phyloseq* package. After first checking that mixed models did not improve fit to the observed data according to Akaike information criterion, diversity patterns were tested for statistical differences across sites (Teruel and Mora orchards) and year of sampling (2017 and 2018) based on ANOVAs ($n = 45$). The assumptions of a normal distribution and constant variance were assessed with residuals plots and Shapiro-Wilk tests. Prokaryotic β-diversity was assessed through a principal coordinate analysis (PCoA) based on Bray Curtis dissimilarity metrics, with the function "ordinate" from the *phyloseq* package. Statistical differences in the observed community composition among sites and years were assessed with a permutational analysis of variance (PERMANOVA) in the R package *vegan* version 2.6.10 with function "adonis2" [43]. For analysis at the OTU or taxon level, read counts were converted to relative abundances by dividing by the total number of reads in the sample, although acknowledging the limitations of these data due to their compositional nature [44].

A co-occurrence network was constructed to explore the relationships within the prokaryotic community in nests ($n = 45$), generated with the Spiec-Easi method in the R package *NetCoMi* version 1.1.0 [45] and plotted with the Fruchterman-Reingold layout from the R package *igraph* version 2.1.4 [46]. Only OTUs with frequency higher than 0.75, and relative abundance higher than 0.001 were included in the analysis, thus leaving 185 OTUs for network construction. The network was characterized with properties of node centrality (degree, eigenvector centrality) and global network measures (edge density, transitivity and average path length). In order to assess the deviation of network properties (average path length and transitivity) from random expectations, we built 1000 random networks according to the *G(n, M)* Erdős-Rényi model and compared the observed value with a non-parametric 95% confidence interval determined with the random networks. We used the "sample_gnm" function in the *igraph* package.

To assess the relationship between *T. melanosporum* and prokaryotic α-diversity, the abundance of *T. melanosporum* mycelium (based on qPCR) was correlated against prokaryotic richness and Shannon's index, using Pearson correlations with a 95% confidence interval, after assessing the normality and linearity assumptions. Mycelium abundance was also correlated against α-diversity measurements of the most abundant prokaryotic Phyla. A canonical correspondence analysis (CCA) was performed with the *vegan* package to explore the relationship between the prokaryotic community composition and the abundance of *T. melanosporum* mycelium in nests ($n = 45$). For this, the relative abundance of OTUs in the samples was correlated with the score of samples for the constrained CCA component, using Pearson's correlation with Bonferroni correction for multiple comparisons.

The relationship between prokaryotic and fungal α-diversity in nests was also assessed with Pearson correlations with a 95% confidence interval. Additionally, a symmetric CoCA was used to explore the relationship between the composition of these communities, with the R package *cocorresp* version 0.4.5 [47]. The CoCA was limited to the most common OTUs to avoid inertia issues linked to high dimensionality of data. Only prokaryotic OTUs with appearance frequencies higher than 0.75, relative abundance more than 0.001 (185), and fungal OTUs with appearance frequency greater than 0.25 (144) were included in the CoCA. Three samples were excluded because no fungal OTUs (fun_OTUs) were detected ($n = 42$).

Results

Prokaryotic community composition

After data processing, amplicon sequencing resulted in a total of 1,376,952 16S clean sequence reads (9,108 OTUs). On average, 26,999 ± 7,219 reads per sample and 2,495 ± 377 OTUs per sample were obtained ($n = 51$). The most abundant phyla were Actinobacteria, Proteobacteria and Acidobacteria in the three soil environments (Fig. 1a). Actinobacteria accounted for 27% of the total number of reads in the peat substrate prior to incorporation in the orchard, 36% in the nests and 46% in the bulk soil. Proteobacteria accounted for 34% in the peat substrate, 30% in the nests and 18% in the bulk soil, and Acidobacteria accounted for 16%, 13% and 17% of the reads respectively. Bacteroidetes were also well represented in the peat substrate (7%) and in nests (9%), but were much lower in the soil (2%). The most abundant bacterial genera in nests were Actinobacteria such as

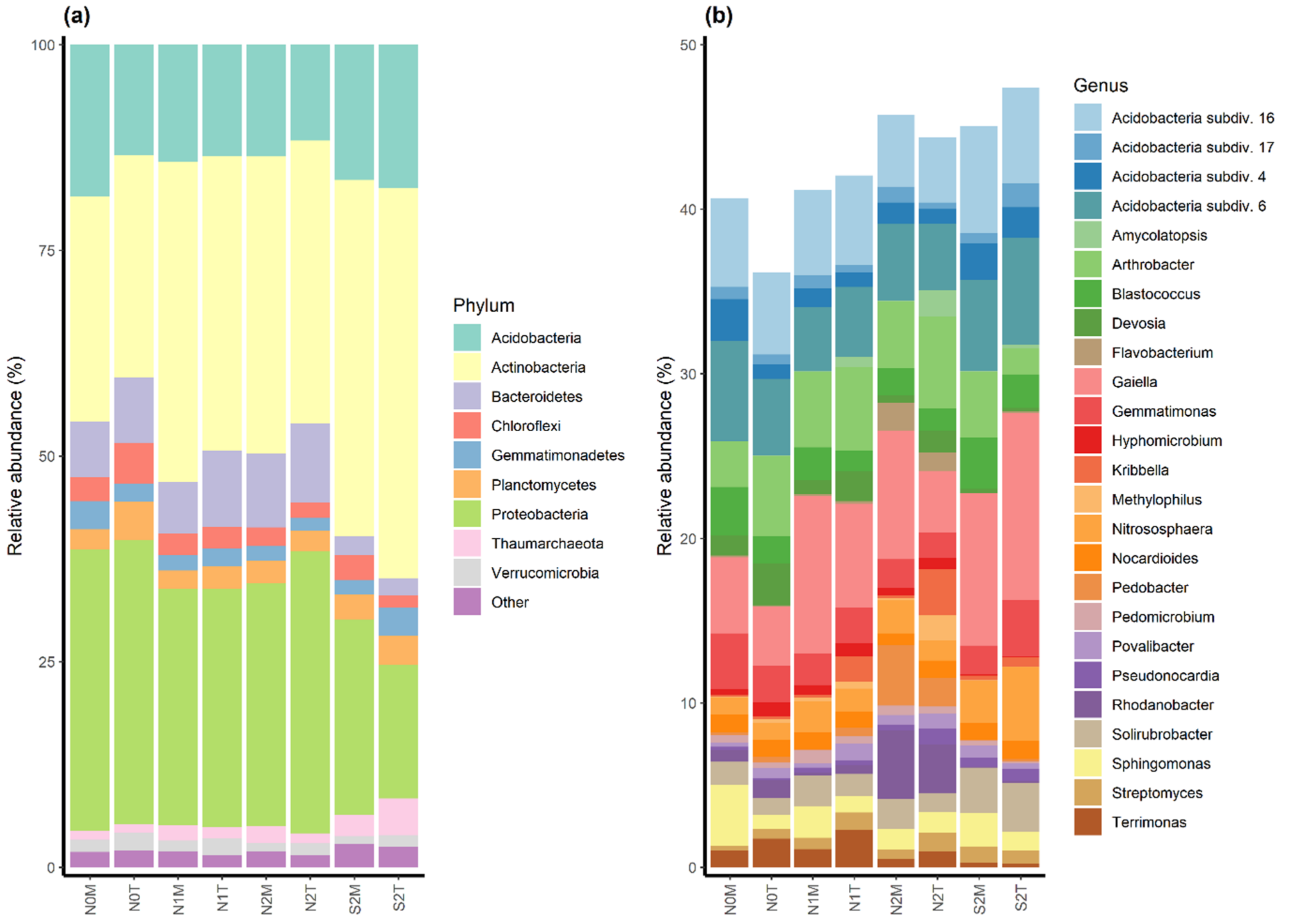

Fig. 1 Stacked bar plot showing the relative abundance (%) of prokaryotic (**a**) phyla and (**b**) genera (including Acidobacteria subdivisions) with relative abundance higher than 1%, for each combination of site and year ($n = 51$ samples). N: nests, S: bulk soil, M: Mora orchard, T: Teruel orchard. The number indicates years from the setting up of the nests (0, 1 and 2 corresponding to years 2016, 2017 and 2018 respectively)

Gaiella, *Arthrobacter*, *Blastococcus* and *Solirubrobacter*, together with Acidobacteria (e.g. chemoheterotrophic members of subdivisions 6 and 16). These genera were also dominant in the peat-based substrate and in the bulk soil of orchards (Fig. 1b, Table S2).

Prokaryotic diversity

In the nests, the richness of prokaryotic OTUs was significantly higher in Teruel than in the Mora orchard (F-value = 4.2, $P = 0.047$, $n = 45$), without significant differences between year 2017 and year 2018 (F-value = 0.2, $P = 0.68$, Fig. 2, Table S3, Fig. S1). No significant differences in the Shannon index were found between sites (F-value < 0.1, $P = 0.96$, $n = 45$), sampling years (F-value = 3.3, $P = 0.076$) or their interaction (F-value = 0.3, $P = 0.57$, Fig. 2). No significant differences in the evenness were found either (F-value = 0.6, $P = 0.45$ for site; F-value = 3.7, $P = 0.061$ for year; F-value = 0.3, $P = 0.56$ for their interaction). The median values of bacterial richness in nests were similar to those in the substrate prior to being incorporated into the orchard soil and tended to be higher than in the bulk soil of the orchards (Fig. 2). The median values of the Shannon index in nests tended to be lower than those in the substrate and tended to be higher than in the bulk soil of the orchards (Fig. 2).

The PERMANOVA analysis of β-diversity showed that there were significant differences in the prokaryotic community composition of nests related to the interaction between the site and sampling year (F-value = 2.1, $P = 0.003$, $n = 45$), with an $R^2 = 0.10$ associated to the site, 0.05 to the sampling year and 0.04 to the interaction (Table S4). This was partially reflected in the PCoA, which did not completely separate the two orchards or the two sampling years, although it showed that Teruel samples tended to cluster around the peat-based substrate prior to being incorporated into the soil, while Mora samples tended to cluster around bulk soil samples (Fig. 3).

Venn diagrams showed that 97.6% of the 3453 OTUs found in the peat substrate ($n = 2$) were also found in the nests ($n = 45$) and 97.7% of the 3579 OTUs found in the bulk soil ($n = 4$) were also found in the nests (Fig. S2a).

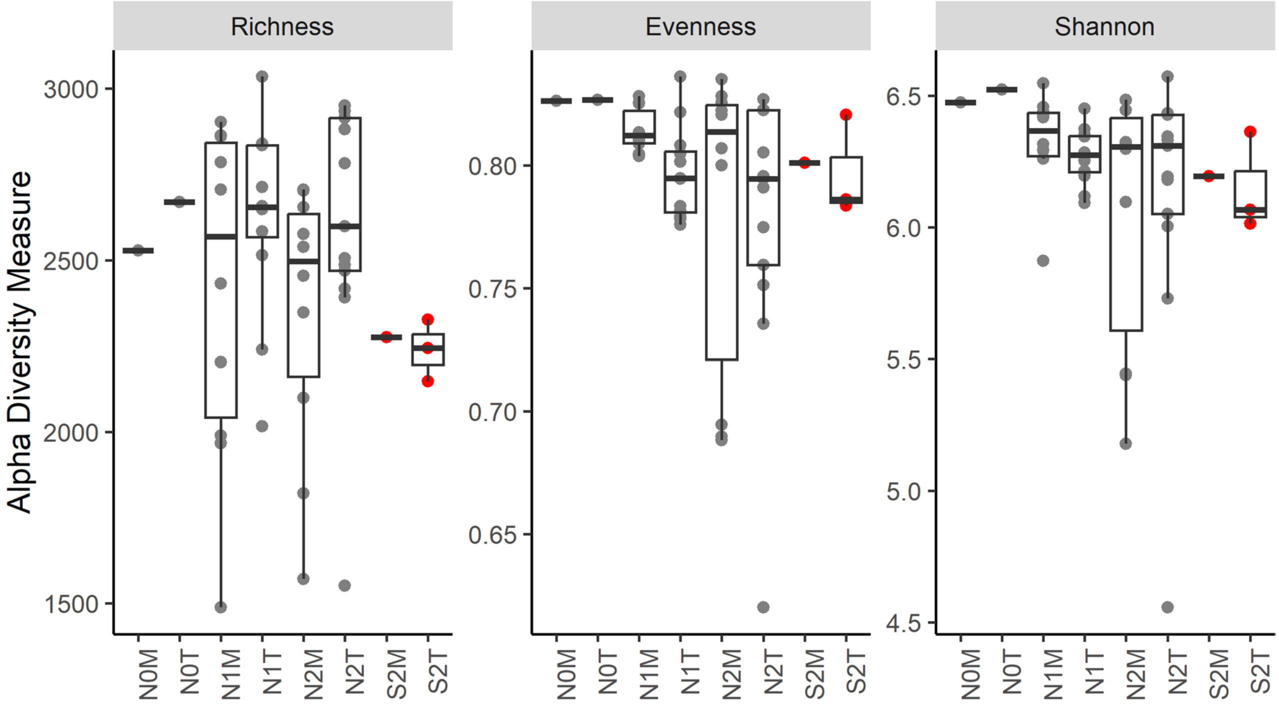

Fig. 2 Boxplot of α-diversity measures (observed richness, evenness and Shannon index) for prokaryote OTUs in the samples (*n* = 51). N: nests, S: bulk soil, M: Mora orchard, T: Teruel orchard. The number indicates years from the setting up of the nests (0, 1 and 2 corresponding to years 2016, 2017 and 2018 respectively). Nest samples in grey and bulk soil samples in red

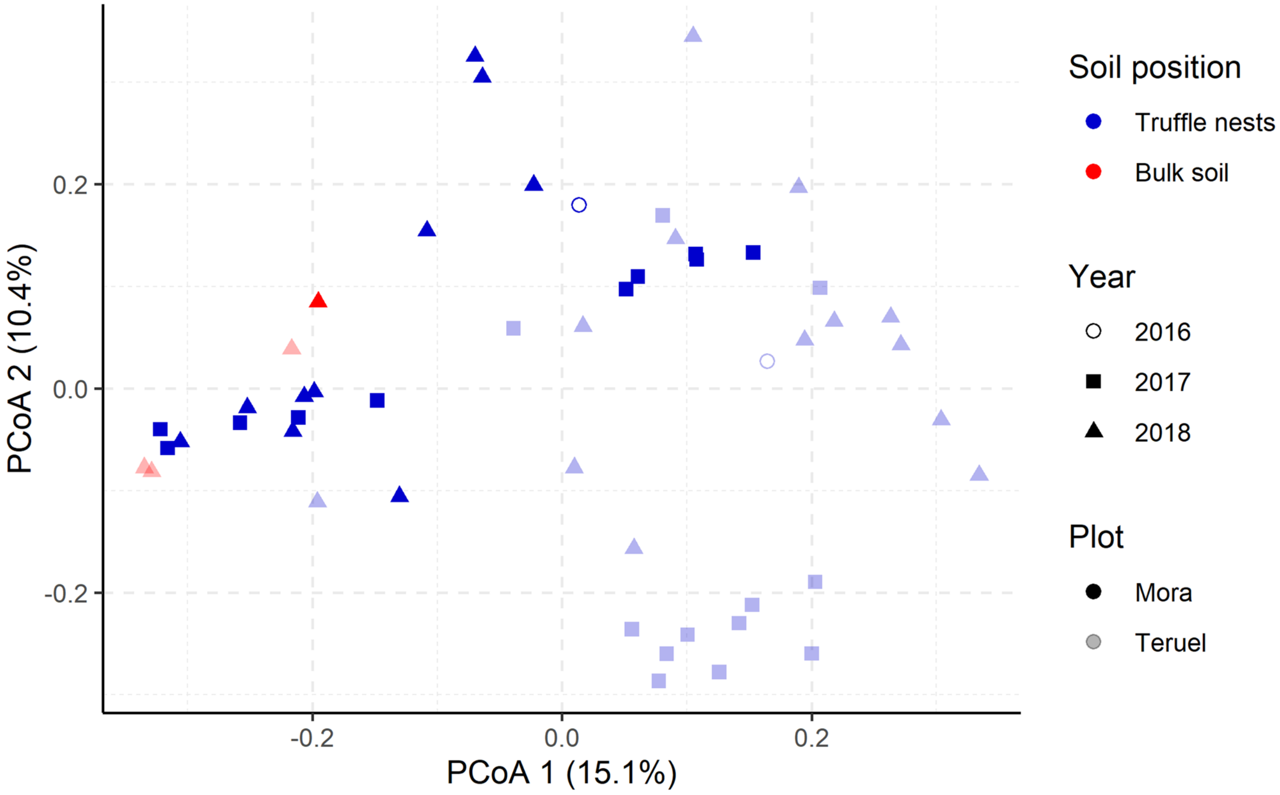

Fig. 3 Principal coordinates analysis plot, using Bray-Curtis dissimilarity matrices, of prokaryotic communities in nests, one and two years after being established (2017 and 2018, respectively) in two truffle orchards (Teruel and Mora) (*n* = 45). Substrate samples prior to being incorporated into the soil (labelled as 2016) and bulk soil samples are also depicted (*n* = 2 and *n* = 4 respectively)

This shared prokaryotic richness was also higher than 90% for all the nest categories (Fig. S2b–e). As a reference, 67.4% of the OTUs found in the peat substrate were also found in the bulk soil.

Prokaryotic co-occurrence network

The network of the filtered bacterial community (185 nodes) presented no singletons, with more than 93% of the nodes having a degree (number of edges) of 4–10 and an average path length (mean shortest distance between each pair of nodes) of 0.999. The edge density of the network was relatively low (0.036), and the transitivity (a global clustering coefficient), was also low (0.157), as typical of relatively sparse networks. Overall, nodes tended to be positively rather than negatively correlated, with positive correlations accounting for 79.2% of the total (Fig. 4a). The average path length and the transitivity of the network were significantly different from the randomly-generated null models (95% confidence interval for the null model average path length: 2.920–2.963 and for transitivity: 0.027–0.046).

The network showed high modularity (0.52) and eight modules (clusters) were identified (Fig. 4a). Four modules presented a central position, with three of them containing the ten main hubs (nodes with highest centrality) according to their eigenvector centrality: OTU17 (order Rhizobiales), OTU50 (genus *Hyphomicrobium*), OTU115 (class Betaproteobacteria), OTU123 (class Alphaproteobacteria), OTU112 (phylum Chloroflexi) and OTU70 (Acidobacteria subdiv. 16) were hubs in a module with 28 nodes; OTU43 (Acidobacteria subdiv. 16) and OTU46 (genus *Dokdonella*) were hubs in a module with 30 nodes; whereas OTU18 (genus *Terrimonas*) and OTU60 (genus *Gemmatimonas*) were hubs in a module with 19 nodes (Fig. 4a). There was another module with 17 nodes in a central position but without hubs. The other four modules were peripheral to those ones and contained 41, 28, 17 and 5 nodes. Proteobacteria, and to a lesser extent Acidobacteria, were dominant in the central modules, whereas Actinobacteria tended to be located in more peripheral modules (Fig. 4b).

Co-occurrence patterns with *T. melanosporum* mycelium abundance

The abundance of *T. melanosporum* mycelium in nests was significantly higher in year 2018 than in 2017 (F-value = 13.6, $P < 0.001$, $n = 45$), without significant differences between the orchard soils (F-value = 2.4, $P = 0.13$, $n = 45$, Fig. 5a). Prokaryotic richness showed a significantly positive correlation with the abundance of *T. melanosporum* mycelium in the nests 1–2 years in the field ($r = 0.35$, $P = 0.018$, $n = 45$), whereas the Shannon index did not (r = -0.01, $P = 0.92$, Fig. 5b, Fig. S3). Among the dominant phyla, these correlations were significant only for Proteobacteria ($r = 0.41$, $P = 0.005$, Fig. 5b, Fig. S3) and among the remaining phyla, only for Verrucomicrobia ($r = 0.40$, $P = 0.006$, Table S5, Fig. S4). Among the Proteobacteria orders with more than 1% relative abundance of the total number of reads, the abundance of *T. melanosporum* extraradical mycelium showed a significant and positive correlation with the richness of Rhizobiales, Xanthomonadales, Rhodospirillales, Burkholderiales and Myxococcales (Table S6, Fig. S4).

According to the CCA, the abundance of *T. melanosporum* mycelium showed a significant relationship with the composition of the prokaryotic community (F = 1.79, $P = 0.038$, constrained inertia: 4.0%). There were 272 OTUs that showed a significant correlation with the CCA constrained component, which was related to truffle mycelium abundance (Fig. 5c). Among them, 180 showed a negative correlation with this CCA component, indicating a positive correlation with *T. melanosporum*. The ones with higher occurrence (appearance frequency higher than 0.75 and relative abundance higher than 0.001) were mainly Alphaproteobacteria (e.g. Rhizobiales belonging to genera *Rhizobium*, *Bradyrhizobium* or *Aminobacter*, OTU37, OTU38, OTU87, OTU89) and Actinobacteria (e.g. Pseudonocardiaceae, OTU14, OTU71, OTU126, Table S7). Meanwhile, 92 OTUs showed a positive correlation with the constrained CCA component, indicating a negative correlation with *T. melanosporum*. Of those, most of the highest occurrences were Actinobacteria, particularly within the genus *Gaiella* (e.g. OTU23, OTU35, OTU40) and the order Solirubrobacterales (OTU9, OTU610; Table S7).

Co-occurrence patterns with the fungal microbiome

In nests that were 1–2 years in the field, prokaryotic richness showed a significantly positive correlation with fungal richness ($r = 0.45$, $P = 0.002$, $n = 45$), whereas the Shannon index of the prokaryotic and fungal communities did not show any significant correlation (r = -0.03, $P = 0.84$, $n = 45$).

According to the CoCA, the common variance between the two microbial groups explained 44.3% of the total variation in the prokaryotic community composition and 20.8% of the variation in the fungal community. The first CoCA component (CoCA1) accounted for 53.6% of the common variance, whereas the second component (CoCA2) accounted for 20.4%. The scores of prokaryotic OTUs were distributed in the CoCA1-CoCA2 biplot following patterns similar to those of the fungal OTUs (Fig. 6), which hints at a relation between the structure of these communities. The fungal scores for the first two CoCA components were highly correlated with those of the prokaryotic community (with Pearson's correlations of 0.80 and 0.77, $P < 0.001$ in both cases).

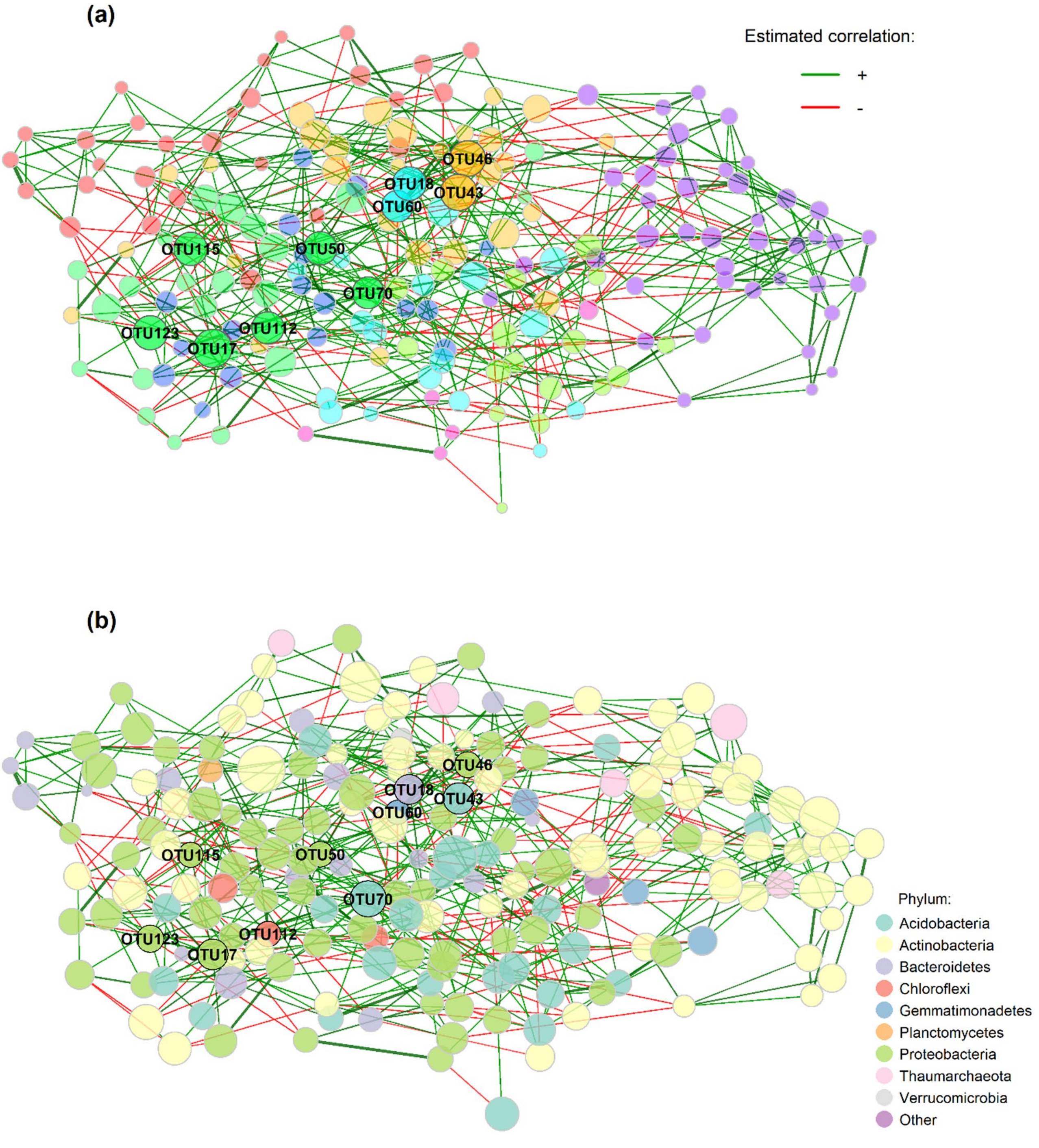

Fig. 4 Prokaryote co-occurrence network in truffle nests. Each node represents an OTU, although the ID is only shown for the hubs. Green edges indicate positive co-occurrences and red edges negative co-occurrences. In **a**, the node colors show the eight clusters in the community, the node size being proportional to the OTU eigenvector centrality. In **b**, the node colors show the taxonomic composition of the network and the node size is proportional to its mclr-transformed relative abundance. Only OTUs with a frequency higher than 0.75 and relative abundance higher than 0.001 were included in the analysis ($n = 185$)

Several of the 2018 samples were positively associated with CoCA1, and their prokaryotic community exhibited higher occurrence of OTUs of Proteobacteria such as *Rhodanobacter* or *Burkholderia* (OTU7, OTU66, OTU108, OTU234), and Sphingobacteriia such as *Pedobacter* (OTU10, OTU62, OTU 369), whereas the fungal community exhibited higher occurrence of several Ascomycota in genera *Pseuderotium* (fun_OTU19), *Nadsonia* (fun_OTU172), *Candida* (fun_OTU1183), *Picoa* (fun_OTU64) and *Penicillium* (fun_OTU241)

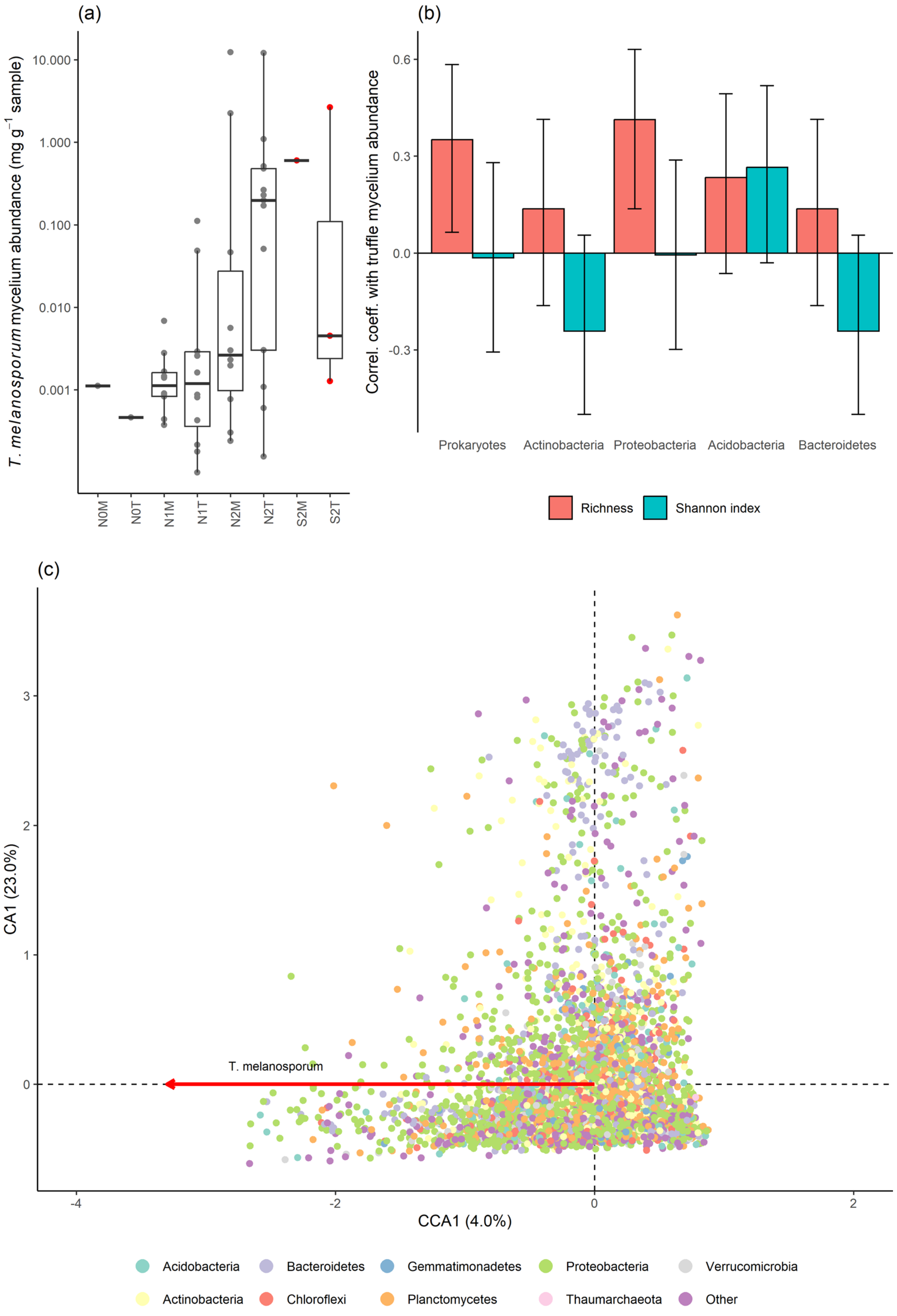

Fig. 5 (See legend on next page.)

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Fig. 5 Abundance of *T. melanosporum* mycelium in nests (**a**) and relationship with the prokaryotic community after 1–2 years in the field: (**b**) Pearson correlation coefficients with prokaryotic α-diversity and the main prokaryotic phyla (mean predicted values and 95% confidence intervals, α = 0.05, *n* = 45); (**c**) Canonical correspondence analysis (CCA) with prokaryotic OTUs. Each point in (**c**) represents an OTU and the red vector represents the increasing abundance of *T. melanosporum* mycelium, which was introduced as environmental variable. In **a**, N: nests, S: bulk soil, M: Mora orchard, T: Teruel orchard, with number 0, 1 and 2 corresponding to years 2016, 2017 and 2018 respectively

(Fig. 6). The Teruel plantation was negatively associated with CoCA2, and its prokaryotic community exhibited higher occurrence of OTUs of several Actinobacteria (OTU3, OTU14, OTU71) and Sphingobacteriia (OTU75, OTU147), whereas the fungal community exhibited higher occurrence of Ascomycota of order Hypocreales (fun_OTU102) and genus *Acremonium* (fun_OTU80) and Basidiomycota of genus *Clitopilus* (fun_OTU332) (Fig. 6). The Mora plantation was positively associated with CoCA2, and its prokaryotic community exhibited higher occurrence of Proteobacteria (OTU86, OTU120), Acidobacteria (OTU166) and Bacteroidetes belonging to genus *Chryseolinea* (OTU82), whereas the fungal community exhibited higher occurrence of Mucoromycota belonging to *Mortierella* (fun_OTU2052) and Basidiomycota belonging to *Tausonia* (fun_OTU32, fun_OTU3285) (Fig. 6).

Discussion

In this study, we characterized the diversity, composition and structure of the prokaryotic communities within truffle nests at two *T. melanosporum* plantations over the initial two-year period following nest establishment. We had hypothesized that the prokaryotic diversity in nests would rapidly change from their first to their second year in the field, and that this would also be reflected in a differentiation from the commercial substrate and an assimilation to the surrounding soil. However, no significant differences were found in the α-diversity between the first and second-year nests, and the temporal differences in β-diversity shown by the PERMANOVA had no strong explanatory power. Contrary to our expectations, we did not find drastic differences in the α- or β-diversity of nests with respect to the commercial substrate prior to being incorporated into the soil or with respect to the bulk soil of plantations (Figs. 2 and 3, Fig. S2a). Thus, these data do not support the hypothesis that nests serve as an open niche that is quickly colonized by pioneer soil bacteria. Rather, the niche is already colonized by bacteria, and the communities may change through ecological drift or other processes. However, comparisons of nests with the commercial substrate are limited by the low sample size of the latter, and comparisons with the bulk soil are also limited by this being sampled only in 2018.

These observations diverge from previous results on the fungal community within these samples, where we found: (i) fungal richness and Shannon index to increase over time following nest establishment, and (ii) fungal richness and taxonomic composition within nests to be clearly differentiated from those in the bulk soil [12]. The contrasting diversity patterns observed between prokaryotic and fungal communities might be attributed to the considerably richer prokaryotic community found in commercial peat compared to its fungal counterpart, although bacterial communities are commonly richer than fungal ones in environmental samples [48]. An alternative explanation could be that the bacterial communities inhabiting the surrounding bulk soil do not colonize the new edaphic niches created by nest establishment with the same efficiency as the fungal microbiota and that bacterial groups present in the peat-based substrate follow temporal patterns that are not noticeable at scales of 1–2 years.

Although prokaryotic α-diversity indices showed minimal variations among soil environments, the data suggested a progressive increase of OTUs belonging to phylum Proteobacteria from bulk soil to nests and further to the commercial substrate, while the phylum Actinobacteria apparently showed the opposite trend (Fig. 1). Moreover, the CoCA analysis showed a positive association of some second-year nests with specific Proteobacteria genera such as *Rhodanobacter* or *Burkholderia*. These findings imply that the substrate may have a legacy effect (or lasting influence) on the taxonomic composition of the prokaryotic community in the nests, although it should be confirmed in other sites with larger sample sizes. Our findings show that Proteobacteria tended to be more central in networks within the prokaryotic community assembly than Actinobacteria (Fig. 4b), potentially indicating their significance in community structuring and functioning [49]. Both Proteobacteria and Actinobacteria have been observed as dominant phyla in truffle orchards not only in our study, but also in various other soil-related studies of various truffle species [8, 14, 50, 51]. In our study, the nests appear to generate a distinctive spatial pattern in the relative occurrence of the main prokaryotic phyla.

The abundance of *T. melanosporum* mycelium showed a positive correlation with the prokaryotic richness, and specifically with Proteobacteria richness. It also showed a positive relationship with the occurrence of specific OTUs, the most abundant of which were Alphaproteobacteria (belonging to genera *Rhizobium*, *Bradyrhizobium* or *Aminobacter*). Conversely, the most abundant OTUs that negatively correlated with *T. melanosporum* belonged to Actinobacteria (Table S7). These findings

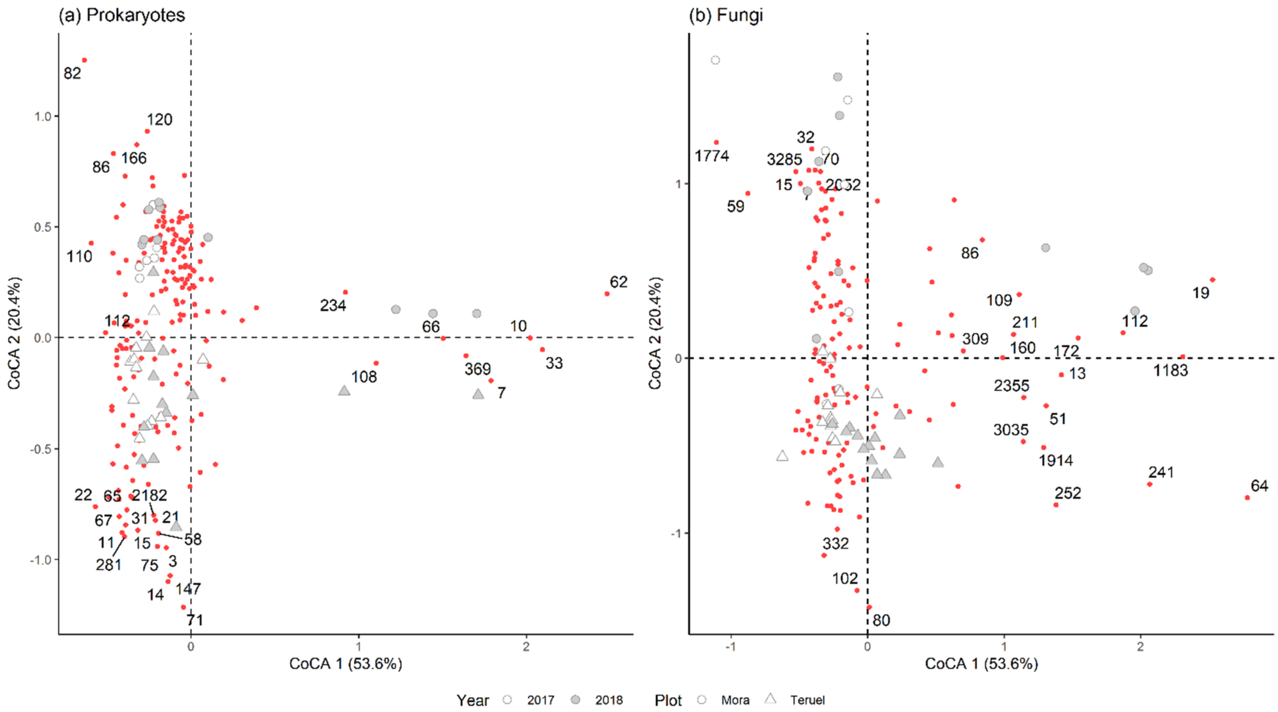

Fig. 6 Biplots for the co-correspondence analysis (CoCA) of prokaryotic (**a**) and fungal (**b**) communities in truffle nests (*n* = 42). Red points represent the position of OTUs on the first two CoCA components and grey shapes represent the samples. Only OTUs with the highest scores for one of the components are labelled with their ID. The analysis only includes the most common prokaryotic (*n* = 185) and fungal (*n* = 144) OTUs

align with previous studies on truffle-associated soils, such as Fu et al. [51], who reported an increase of Proteobacteria in *T. indicum*-producing soils with respect to control soils, and Wang et al. [52], who observed a decrease in Actinobacteria.

Previous studies on truffle ascocarp microbiome showed an enrichment of Proteobacteria (and frequently a reduction in Actinobacteria) with respect to the surrounding soil, leading to the hypothesis that ascocarps create a niche that selectively favors specific prokaryotic communities [8, 53–55]. A comparable scenario has been described for truffle mycorrhizae and for the ectomycorhizosphere of other ecosystems, although some studies have reported an increase of Actinobacteria in truffle mycorrhizosphere [14, 15, 56, 57]. Our results suggest a local increase of the relative abundance of Proteobacteria within the nests and a positive correlation between nest colonization by *T. melanosporum* and Proteobacteria richness, which could potentially exert a synergistic effect.

The positive association of Proteobacteria with both nests and truffle mycelium appears to be driven by specific taxa, such as orders Rhizobiales (*Aminobacter*, *Bradyrhizobium*, *Rhizobium*), Xanthomonadales (*Rhodanobacter*), Rhodospirillales (*Reyranella*) or Burkholderiales (*Burkholderia*), as well as genera *Caulobacter* and *Phenylobacterium* (order Caulobacterales). Previous studies have emphasized the occurrence of Rhizobiales, particularly *Bradyrhizobium* and, to a lesser extent, *Rhizobium* and *Aminobacter*, in ascocarps and mycorrhizae of different truffle species [8, 16, 54, 56, 58]. This has led to the hypothesis that certain rhizobia play essential roles in the formation and/or functioning of these fungal structures, specifically in key processes such as nitrogen fixation, phosphate limitation, resistance to metals and oxygen stress, or sulfur oxidation [8, 59–61]. Furthermore, other studies have reported a positive interaction of Rhizobiales with truffle mycelium growth [62, 63]. Despite the important ecological implications of these tripartite relationships between plant, mycorrhizal fungi and bacteria, they remain underexplored, with mycorrhizosphere bacteria adversely affecting fungal activity in some cases and exerting positive effects on soil nutrient cycles, plant growth, plant stress alleviation and mycorrhiza formation in other cases [64].

Burkholderia has also been reported to be dominant within *T. magnatum* ascocarps [65], while Antony-Babu et al. [8] reported a high prevalence of *Variovorax*, a genus from the same order (Burkholderiales), in *T. melanosporum*. Interestingly, *Variovorax* was the most abundant genus of Burkholderiales in sampled nests and soils (Fig. S4). Deveau et al. [15] found that orders Caulobacterales, Rhodospirillales, Rhizobiales and Xanthomonadales were more abundant in *T. melanosporum* mycorrhizae compared to the surrounding soil. Furthermore, Li et al. [66] and Zhang et al. [67] found that *Reyranella* was enriched in *T. indicum* mycorrhizospheric soil, while Ma et al. [68] found that Proteobacteria genera such as *Phenylobacterium*, *Dokdonella*, *Bradyrhizobium* or *Rhodanobacter* were indicators of *T. sinense* producing soils. Kang et al. [61] observed that the families Rhizobiaceae, Bradyrhizobiaceae, Burkholderaceae and Rhodanobacteraceae were major components in the denitrifier community within the mycorrhizosphere of *Tuber*. Zhang et al. [60] also pointed to the potential involvement of Xanthomonadales in phosphorus cycling. Despite these findings, the role of these bacteria in multipartite symbiotic relations and the implications on the truffle life cycle remain unclear and warrants further investigation, which could be addressed with a functional characterization of these communities [8].

In the nests studied here, the factor referring to the geographical origin and characteristics of the plantation exerted a more significant influence on the prokaryotic β-diversity compared to time. We observed a higher occurrence of several Proteobacteria OTUs in the nests of the Mora plantation compared to Teruel. These differences between plantations could be attributed to a combination of factors, such as soil physicochemical properties and management practices including irrigation or soil tilling. Previous research has shown that both soil properties and agronomic management can affect soil prokaryotic communities [69, 70]. In this regard, Khan et al. [71] found that the retention and incorporation of plant debris from mulch into the soil under conventional tillage increased the relative abundance of members of Proteobacteriota, while other studies have also pointed out an increase of Alphaproteobacteria in tilled soils [72, 73]. However, it remains challenging to assess how compositional changes in nests could affect truffle fruiting. Future experimental studies may explore the effects of various substrates or substrate supplementation with specific bacteria on community composition or structure, and whether such modifications could lead to improved truffle yields with respect to standard practice.

Our study revealed a positive correlation between the prokaryotic and the fungal community richness in the nests, along with common patterns of variation between both communities (Fig. 6). This finding adds to the positive correlation between truffle mycelium abundance and prokaryotic richness of specific phyla and orders (Fig. 5b, Table S5), highlighting the significance of positive interactions in nests. Previous studies with *T. magnatum* and *T. indicum* have also found that prokaryotic α-diversity did not decrease in truffle-producing soils with respect to control soils, despite the changes in the taxonomic composition [51, 65]. Additionally, network analysis showed that in our nests, positive correlations among prokaryotic OTUs were more prevalent compared to *T. indicum* natural soils [55]. We had hypothesized that the prokaryotic α-diversity would be negatively affected by fungi. On the contrary, our results collectively indicate that nests constitute a habitat where positive bacterial-fungal or positive bacterial-bacterial associations dominate the bacterial community structure [20, 21]. These findings stand in contrast to the previously reported negative effect of *T. indicum* colonization on the prokaryotic diversity in rhizospheric soil [67].

In conclusion, we characterized the bacterial community of truffle nests and assessed the ecological relationships of soil bacteria with the abundance of truffle mycelium and the soil fungal community. Contrary to our hypothesis that nests serve as an open niche for pioneer bacteria colonization, nests were already well colonized by diverse bacteria and we did not observe a rapid change in the prokaryotic α- or β-diversity of nests in the field, nor did we observe clearcut differences with the surrounding soil. We found that Proteobacteria and Actinobacteria were dominant in the prokaryotic communities of nests, with a relative increase in relative abundance of the former and a decrease in the latter relative to that in the surrounding soil. Additionally, the abundance of *T. melanosporum* mycelium in the nests was positively correlated to Proteobacteria richness and the abundance of several OTUs, many belonging to this phylum. The positive association between *T. melanosporum* and specific taxa of Proteobacteria, combined with the positive relationship of this phylum with nests, indicates a possible underlying factor for the performance of nests in truffle plantations.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40793-025-00848-6.

Supplementary Material 1

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Author contributions

Pedro Marco (Conceptualization, Funding acquisition, Investigation, Methodology, Writing—original draft), Sergio Sánchez (Conceptualization, Funding acquisition, Investigation, Methodology, Writing—review & editing), Sergi Garcia-Barreda (Conceptualization, Formal analysis, Methodology, Writing—original draft), Javier Parladé (Funding acquisition, Investigation, Writing—review & editing), Mara Rondolini (Formal analysis, Writing—review & editing), Vicente González (Writing—review & editing), Gian Maria Niccolò Benucci (Conceptualization, Formal analysis, Investigation, Methodology, Writing—original draft), Gregory Bonito (Funding acquisition, Investigation, Methodology, Writing—review & editing).

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Data availability

The 16S rRNA gene amplicon sequencing data have been deposited in the NCBI Sequence Read Archive (SRA) and can be accessed under the BioProject accession number PRJNA1255533 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1255533). The ITS amplicon sequencing data are available under the BioProject accession number PRJNA938598 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA938598). The *Tuber melanosporum* mycelium qPCR data are openly available in FigShare at https://doi.org/10.6084/m9.figshare.29890877. To reproduce the analysis performed in this study, data sets and R code are openly available at https://github.com/Gian77/Published-R-Code/tree/master/Garcia-Barreda_et_al_TruffleNestBacteria.

Declarations

Competing interests

The authors declare no competing interests.

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