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Title: Microbial richness and composition independently drive soil multifunctionality

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Running title: Microbial drivers of soil multifunctionality.

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Abstract

1. Soil microbes provide multiple ecosystem functions such as nutrient cycling, decomposition and climate regulation. However, we lack a quantitative understanding of the relative importance of microbial richness and composition in controlling multifunctionality. This knowledge gap limits our capacity to understand the influence of biotic attributes in the provision of services and functions on which humans depend.
2. We used two independent approaches (i.e. experimental and observational), and applied statistical modeling to identify the role and relative importance of bacterial richness and composition in driving multifunctionality (here defined as seven measures of respiration and enzyme activities). In the observational study we measured soil microbial communities and functions in both tree- and bare soil-dominated microsites at 22 locations across a 1200 km transect in southeastern Australia. In the experimental study we used soils from two of those locations and developed gradients of bacterial diversity and composition through inoculation of sterilized soils.
3. Microbial richness and the relative abundance of γ-Proteobacteria, Actinobacteria and Bacteroidetes were positively related to multifunctionality in both the observational and experimental approaches; however, only Bacteroidetes was consistently selected as a key predictor of multifunctionality across all experimental approaches and statistical models used here. Moreover, our results, from two different approaches, provide evidence that microbial richness and composition are both important, yet independent, drivers of multiple ecosystem functions.
4. Overall, our findings advance our understanding of the mechanisms underpinning relationships between microbial diversity and ecosystem functionality in terrestrial ecosystems, and further suggest that information on microbial richness and composition needs to be considered when

formulating sustainable management and conservation policies, and when predicting the effects of global change on ecosystem functions.

Key words: Bacteria, Enzyme activities, BEF relationship, Nutrient cycling, Terrestrial ecosystems

Introduction

The status of Earth's biodiversity is in decline (Dirzo *et al.* 2014). The loss of species has global consequences because biodiversity promotes ecosystem functions and services that are essential for human well-being (Hooper *et al.* 2005; Cardinale *et al.* 2012). These services include food production, nutrient cycling and climate regulation; and have been valued at trillions of U.S. dollars per year (Costanza *et al.* 1997). The importance of biodiversity for ecosystem functions and services has been shown (Cardinale *et al.* 2011; Tilman *et al.* 2014), however, biodiversity is extremely complex, and involves different components including, but not limited to, species richness (number of taxa) and composition (i.e. identity of the different organisms comprising a community expressed in terms of their relative abundance; Díaz *et al.* 2001). Both taxa richness and composition have been reported to influence one or several ecosystem functions (Díaz *et al.* 2001; Hooper *et al.* 2005; Flynn *et al.* 2011; Isbell et al. 2011; Allan *et al.* 2013; Dooley et al. 2015; Lefcheck & Duffy 2015). Variation in composition can act in synergy or opposition to effects of richness in natural (rather than randomly assembled experiments) systems, and thus, the role of different aspects of diversity (composition, richness, identity) remains unclear (Wardle *et al.* 1999; Leps *et al.* 2001; 2004). Moreover, the relative importance of these two biodiversity metrics for increasing the provision of several ecosystem processes simultaneously (multifunctionality) remains largely unexplored (Isbell *et al.* 2011; Byrnes *et al.* 2014a,b; Dooley *et al.* 2015). Both species richness and composition are likely to change markedly under future climatic scenarios or more intense land uses (Díaz *et al.* 2001; Hooper *et al.* 2005). Therefore, it is critical that we quantify the relative importance of these biodiversity components for multifunctionality so that we can formulate appropriate management and conservation policies and predict the likely changes in ecosystem functioning under changing environments.

Unlike plants or animals (Hooper *et al.* 2005; Lefcheck *et al.* 2015), we have only a limited understanding of the relationships between microbial diversity and composition, and ecosystem functioning, particularly in terrestrial environments (Bardgett & van der Putten 2014). Microbes are considered by far the most abundant and diverse life forms on Earth (Singh *et al.* 2009), and play essential roles in maintaining multiple ecosystem functions including litter decomposition, primary production, soil fertility and gaseous emissions (He *et al.* 2009; Peter *et al.* 2011; Jing *et al.* 2015; Delgado-Baquerizo *et al.* 2016). Global environmental drivers such as land use change, nitrogen enrichment and climate change are impacting upon both soil microbial diversity and composition (Wall *et al.* 2010; Gans *et al.* 2005; Maestre *et al.* 2015). In order to evaluate the global consequences of shifting microbial diversity on multifunctionality, it is critical that we account for the independent effects of species richness and composition on multiple ecosystems functions (Downing & Leibold 2002; Hooper *et al.* 2005).

A growing body of experimental and observational studies suggests that microbial diversity promotes ecosystem multifunctionality in terrestrial and aquatic ecosystems (He et al. 2009; Peter et al. 2011; Jing *et al.* 2015; Delgado-Baquerizo *et al.* 2016). For example, Peter et al. (2011) provided experimental evidence for a link between microbial richness and ecosystem multifunctionality in bacterial aquatic biofilms. Moreover, using field surveys, He et al. (2009), Jing *et al.* (2015) and Delgado-Baquerizo *et al.* (2016) found strong positive relationships between microbial alpha diversity and multifunctionality from local to global scales. Much less is known, however, of the role of microbial composition in driving multifunctionality. Recently, whole genome sequencing (Trivedi *et al.* 2013) has provided evidence that dominant bacterial groups such as Actinobacteria phyla and Proteobacteria classes (e.g. γ-Proteobacteria) can potentially play different roles in supporting critical ecosystem processes such as decomposition and nutrient cycling. However, despite these findings, we still lack empirical evidence from either observational or manipulative studies of the roles of these microbial taxa in supporting multifunctionality in terrestrial ecosystems. Only recently, studies based on plant communities have started explicitly considering the simultaneous effects of both plant composition and diversity in driving multifunctionality (Isbell et al. 2011; Dooley et al. 2015; Lefcheck & Duffy 2015) Conversely, to the best of our knowledge, no study has statistically evaluated the relative importance of soil microbial richness and composition (i.e. relative abundance of main phyla and classes) in controlling multifunctionality. Assessing the relative importance of microbial diversity and composition in driving multifunctionality is critical to include microbial communities and processes in ecosystem and earth system simulation models, and to consider their status when making policy or management decisions.

Herein, we combined a regional field survey and a microcosm experiment manipulating the diversity of bacteria in two soils to identify the role and relative importance of microbial richness and composition in predicting multifunctionality. We hypothesized that microbial richness and composition are both important, but operate independently, as drivers of terrestrial multifunctionality. Our rationale is that microbial richness and composition represent two different mechanisms controlling multifunctionality. First, for microbial interaction (complementarity effects; Loreau & Hector 2001): theoretical frameworks (Schimel *et al.* 2005) predict that complex processes such as chitin degradation (Beier & Bertilsson 2013), require a large and diverse group of microbes. Second, regarding microbial identity: whole genome sequencing information indicates that different microbial groups can potentially play idiosyncratic roles in ecosystem processes such as organic matter decomposition and nutrient cycling, which may potentially affect the rates in which these processes are being produced (Floudas *et al.* 2012; Trivedi *et al.* 2013).

Material and Methods

Study sites and soil sampling.

We used two independent but complementary approaches to evaluate the role and relative importance of microbial richness and composition in supporting multifunctionality: an observational study that utilized a broad regional soil survey (Field survey), and an experimental microcosm approach (Microcosm study). Note it is not our intention to directly compare results between experimental approaches. Rather, our goal is to address our research question by using two very different, but complementary, approaches (experimental and observational studies) and thus provide further rigorous scientific support to our findings. We define microbial richness as the number of taxa (microbial phyla/classes) and microbial composition as the identity of the different microbial taxa comprising the soil community (in an environmental soil sample or microcosm), expressed in terms of relative abundance.

Rationale of the use of observational and experimental approaches to identify the role of microbial richness and composition in controlling multifunctionality.

Observational data (e.g. changes along a broad environmental gradient) provide useful information on how bacterial diversity and composition relate to multifunctionality under “real world” scenarios. However, because of the observational nature of this approach, results are correlative and potentially non-causative. Conversely, using an experimental, laboratory-based microcosm with cultures provides a unique opportunity to manipulate both bacterial richness and composition, generating multiple combinations of these two biotic features. The use of cultures alone, however, is usually considered unrealistic because the majority of bacterial taxa are unculturable and there are difficulties in assembling bacterial communities *de novo* (Hooper *et al.* 2005; Bell *et al.* 2005). Culturing is useful however for comparing the results with other ecological studies (Loreau & Hector 2001; Hooper *et al.* 2005). Using both observational and microcosm experimental studies gives us a unique opportunity to separate the differential effects of taxa richness and composition on multiple ecosystem functions.

Field survey (observational approach).

Our observational study was carried out in 22 sites from eastern Australia across a gradient of about 1200 km (Fig. S1; Table S1). Locations were intentionally chosen to represent a wide range of climatic and soil property conditions. Mean annual precipitation ranged from 280 mm to 1167 mm and temperature from 12.8º C to 17.5ºC. Soil organic carbon (soil carbon) and pH ranged from 0.8 to 12.3% and from 4.8 to 9.0, respectively (Table S1). Soil sampling was carried out in March 2014. At each site, three soil cores (0-5 cm depth) were collected from two microsites: under trees (*Eucalyptus* spp.) and in open (bare soil) -dominated sites. Soil cores were then mixed to obtain a composite sample for each microsite at each site. A total of 44 soil samples (22 sites x 2 microsites) were analysed in this study. Following field sampling, the soil was sieved (<2 mm mesh). A portion of the soil was immediately frozen at -20ºC for characterizing bacterial abundance, composition and diversity. The other fraction was air-dried and stored before functional analyses. This storage approach is well established and commonly used when analyzing soil variables such as those evaluated here in large-scale surveys (Maestre *et al.* 2012; Tedersoo *et al.* 2014).

Soil DNA was extracted from 0.25 g of defrosted soil samples using the Powersoil® DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA). We quantified the abundance of total bacteria in all soil samples (Field and Microcosm studies) using 96-well plates on a CFX96 Touch™ Real-Time PCR Detection System (Foster city, California, USA). Bacterial 16S rRNA gene was amplified with the Eub 338-Eub 518 (Lane 1991) primer set as described in Maestre *et al.* (2015). We characterized bacterial diversity and composition in the soil surface (top 5 cm) along our observational gradient by using the Illumina Miseq profiling of ribosomal genes (Illumina Inc.) and the 341F/805R (Herlemann *et al.* 2011) primer set (see details in Appendix S1).

Microcosm study (Experimental approach).

In parallel with the sampling protocol described above, we collected a greater mass of soil (~5kg) from two sites of contrasting aridity and total soil carbon (Soils A and B; Fig. S1; JM072-TREE and Site 1-TREE in Table S1). Soil A had a lower soil carbon than Soil B (3.03% vs. 8.45%). In addition, Soil A had a higher pH than Soil B (6.36 vs. 5.63; Table S1). In both cases, soil samples were collected from under tree canopies. Following field sampling, the soil was sieved (<2 mm mesh), one part stored immediately at 4ºC (non-sterile soil used for the microbial inoculums), and the other sterilized using gamma radiation (50kGy; Appendix S1).

The richness treatment consisted of one, two, four and six bacterial taxa per microcosm. For each of these richness levels, we prepared all the possible equally distributed taxa combinations. A total of 37 (6+15+15+1 combinations corresponding to richness levels one, two, four and six) treatments were prepared per soil. We duplicated the level "six" of diversity to improve the balance of this treatment and to ensure the success of this important level of diversity (6+15+15+2). In addition, and to reduce the correlation between diversity and composition in our experiment, we also prepared additional microcosms with diversity 'two' but with 75/25% and 25/75% of bacterial composition to reduce correlation between taxa richness and composition. This is a critical point, as most previous biodiversity research has not adequately separated composition effects from richness effects due to experimental design constraints (Huston, 1997; Allison, 1999; Hooper *et al.* 2005). This provided 30 new treatments per soil (Table S2). A total of 67+1 combinations were used in this study (a complete list of combinations is shown in Table S2). To ensure the success of our inocula, we established three microcosms for each combination (68 x 3), resulting in a total of 204 microcosms per soil (Soils A and B).

Bacterial strains from six terrestrial dominant phylogenetic taxa belonged to phylum Actinobacteria, Firmicutes, Bacteroidetes, and Proteobacteria classes α-Proteobacteria, β-Proteobacteria, and γ-Proteobacteria (Fig. S2), were isolated across both Soils A and B (Appendix S1 for isolation details and rationale of the selection of these phyla/classes).

Sterile soil samples (10 g) were placed in hermetic containers. Soil samples were inoculated to achieve a total amount of 10^8 cells per microcosm. Thus, the final cell densities in all microcosms were the same, that is, the six-taxa assemblage had the same number of cells (1/6 of each strain) as those in the single taxon assemblage. These microcosms were positioned in a laminar flow cabinet to avoid contamination. Microcosms were incubated in the darkness at 50% soil water content (SWC) and 25ºC for 8 weeks under sterile conditions. Soils were opened to the air every 5 days in a laminar flow cabinet to prevent the samples becoming anaerobic. After incubation, a portion of the soil was immediately frozen at -20 ºC, and the abundance of different bacterial taxa determined using quantitative PCR (qPCR). This step is critical as it provided us with information on the degree to which the original microbial combinations were maintained in our microcosms. The other fraction was used to assess multiple ecosystems functions as described below. Soil DNA extraction and bacterial 16S rRNA gene quantification were done as explained above (i.e., Field survey).

To check whether the original composition assigned to the different microcosms was maintained by the end of the experiment and take into account changes in bacterial abundance in our microcosms, we quantified the abundance of each of Actinobacteria, Bacteroidetes and α-, β- and γ-Proteobacteria and Fimicutes using qPCR (Appendix S1). Both original assigned (when microcosms were constructed) and corrected (after qPCR analyses) relative abundances of bacteria were highly related (Spearman ρ >0.935; *P*<0.001 in all cases) so we used the corrected values in further analyses.

Rationale for the selection of high bacterial taxonomic ranks: phyla/classes.

Our decision to use high bacterial taxonomic ranks to explore the role of microbial richness and composition in controlling multifunctionality (i.e. Microcosm study) is based on three main reasons: (1) the main phyla/classes are globally distributed and common across samples (e.g. Ramirez *et al.* 2012); (2) The use of high bacterial taxonomic ranks (phyla and classes) has been highly recommended to predict patterns in ecosystem functioning (Philippot *et al.* 2010; Trivedi *et al.* 2013); (3) functional information has become increasingly available at this taxonomic level (Fierer *et al.* 2007; Bastian *et al.* 2009; Trivedi *et al.* 2013). This is critical, as understanding how changes in taxa richness and composition influence ecosystem functions requires an understanding of the functional characteristics of the taxa involved (Hooper *et al.* 2005).

Rationale for the selected phyla/classes.

We selected *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and α-,β- and γ-*Proteobacteria* for three main reasons: (1) All of these bacterial taxa are globally distributed and dominant in many terrestrial ecosystems worldwide (Fierer *et al.* 2009; Maestre *et al.* 2005); 2) the selected taxa are all easy to culture under laboratory conditions (see *Microbial isolation* below); and 3) quantitative PCR (qPCR) specific primer sets are available for all these bacterial taxa (i.e. see Microcosm study below).

Measurement of individual ecosystem functions.

In all soil samples, we measured seven variables (hereafter functions): activity of β-glucosidase (starch degradation), cellobiosidase (cellulose degradation), N-Acetylglucosaminidase (chitin degradation), phosphatase (phosphorus mineralization), basal respiration and glucose and lignin induced respiration. Extracellular soil enzyme activities: β-glucosidase, cellobiosidase, N-Acetylglucosaminidase and phosphatase were measured from 1g of soil by fluorometry as described in Bell *et al.* (2013). In addition, we used the Microresp® approach from Campbell *et al.* (2003) to measure basal respiration and glucose and lignin-induced respiration. For the Field study, soil samples were pre-incubated at 50% SWC and 20ºC during five days prior to MicroResp® analyses (Garcia-Palacios *et al.* 2011). Samples with (glucose and lignin) and without (basal respiration) substrates were incubated for 6 h and read at 570 nm. Substrate induced respiration of glucose and lignin are calculated as respiration in glucose or lignin less the basal respiration. Altogether, the selected soil variables (hereafter functions) constitute a good proxy of nutrient cycling, organic matter decomposition, biological productivity, and buildup of nutrient pools (Campbell *et al.* 2003; Schade & Hobbie 2005; Perroni-Ventura *et al.* 2009; Jax 2010; Maestre *et al.* 2012; Bell *et al.* 2013; Bradford *et al.* 2014; Jing *et al.* 2015). Extracellular enzymes such as β-glucosidase, cellobiosidase, N-Acetylglucosaminidase and phosphatase are produced by soil microbes, and are involved in the processing, stabilization, and destabilization of soil organic matter and nutrient cycling in terrestrial ecosystems (Bell *et al.* 2013). They are also considered a good indicator of nutrient demand by soil microorganisms (Bell *et al.* 2013). In addition, basal respiration and glucose induced respiration have been used as a proxy of microbial activity in soil, while lignin degradation provides a metric of the capacity of a particular microbial community to degrade recalcitrant carbon (Campbell *et al.* 2003).

Assessing multifunctionality.

We used three complementary approaches to evaluate the role of microbial diversity and composition in driving multifunctionality: averaging multifunctionality, multiple-threshold method of Byrnes *et al.* (2014a) and multiple single functions. These multifunctionality indexes were independently obtained for the soils in Field and Microcosm studies and also for the Soils A and B in the Microcosm study. It is important to clarify that our intention is not to merge these two soils included in the Microcosm study, but to ensure that our hypotheses are valid after using different experimental approaches and two soils with different soil properties. To obtain an averaging multifunctionality index for each sample, we first normalized (log-transformed when needed) and standardized each of our seven ecosystem functions using the Z-score transformation as described in Maestre *et al.* (2012). Following this, the standardized ecosystem functions were averaged to obtain a multifunctionality index (Maestre *et al.* 2012). Averaging multifunctionality is widely used in the multifunctionality literature and provides a straightforward and easy-to-interpret measure of the ability of different communities to sustain multiple functions simultaneously (Maestre *et al.* 2012; Wagg *et al.* 2014; Bradford *et al.* 2014; Jing *et al.* 2015). However, we stress that the averaging multifunctionality approach explained above also has some limitations. For example, the averaging approach cannot distinguish between (1) two functions having similar values and (2) one function having high values compensating for a second function with low values (Byrnes *et al.* 2014a). To overcome these limitations, we also estimated multifunctionality using the multiple-threshold method of Byrnes *et al.* (2014a), which evaluates the number of functions that simultaneously exceeds multiple critical thresholds. In brief, this approach calculates the maximum value of each measured function and counts the number of functions that exceed a pre-established threshold. For our analyses, we used predetermined thresholds (Byrnes *et al.* 2014a; Bradford *et al.* 2014). Here, we selected three thresholds (25, 50 and 75%) that cover the whole spectrum. This method provides information about the threshold in which our variable maximizes the effect on the number of functions beyond that threshold. In our case, these thresholds inform about the functional level in which more functions are maximized with richness increments and shifts in composition. Our averaging multifunctionality index was highly related to the number of functions at or above 25, 50 and 75% thresholds of the maximum observed function, supporting the appropriateness of our approach ($P<0.001$; Table S4). Thus, for simplicity, we conducted the main analyses in this study using the multifunctionality averaging approach.

<u>*Statistical analyses.*</u>

Exploring the relationship between bacterial diversity/composition and multifunctionality.

For the Field survey (non-replicated approach), we first explored the relationship between bacterial richness and composition (α-, β- and γ-Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria) with multifunctionality and each single function by fitting linear multiple regressions. In addition, we conducted partial correlations between bacterial richness and composition with multifunctionality accounting for latitude/longitude and total bacterial abundance (qPCR) to take into account any bias derived from these important factors. Bacterial diversity was x^2-transformed to improve normality before these analyses.

For the Microcosm study (replicated approach), we examined the effects of diversity on multifunctionality by conducting a nested ANOVA, with diversity as a fixed factor and bacterial combination (Table S1) as a random factor nested within diversity (Quinn & Keough 2002). We repeated these analyses using bacterial abundance as a covariate (ANCOVA) to account for any bias derived from a potential shift of bacterial yield in our microcosms. We then used Spearman's correlations to explore the relationship between the relative abundance of the main bacterial phyla/classes with single functions, averaging multifunctionality and with the number of functions at or above 25, 50 and 75% thresholds of the maximum observed function. Finally, we evaluated the effects of each bacterial phyla/classes identity in supporting multifunctionality in both mono- and mixed cultures (i.e. presence or absence of each taxon across all microcosms) by conducting ANOVA analyses.

Distance-based multimodel inference

To identify the relative importance of richness and composition of bacteria (α-, β-, γ-Proteobacteria, Actinobacteria, Bacteroidetes and Fimicutes) as drivers of multifunctionality, we used a multi-model inference approach based on information theory and non-parametric distance-based linear regressions (DISTLM; McArdle and Anderson 2001). We did these analyses using the PERMANOVA+ for PRIMER statistical package (PRIMER-E Ltd., Plymounth Marine Laboratory, UK). The Euclidean distance was used as the measure of multifunctionality dissimilarity between pairs of samples. Bacterial richness represents the number of inoculated phylotypes in the case of our Microcosm study, and the number of OTUs (species) of all bacteria in the case of our Field survey. In the Microcosm study, the composition of bacteria represents the relative abundance of the six inoculated taxa. In the case of the Field survey we used two approaches to represent the composition of bacteria including: (1) relative abundance of the six selected taxa (those in our experimental approach) accounting for 28-74% (average 53%) of the relative abundance of all bacteria. Thus our aim was to directly compare results from our field and experimental approaches; and (2) a representation of the composition of the entire community of bacteria (100% of species) (using the axes from a NMDS). To obtain a metric of community composition at the lowest taxonomic rank, we used a non-metric multidimensional ordination (NMDS) on the matrix of bacterial composition at the OTU level (i.e. species level). Given a low stress in these analyses (0.05), the axes of a NMDS are considered a good representation of the variation in the composition of entire bacterial communities across samples. We kept the three-dimensional NMDS solution for further analyses. We conducted NMDS ordinations with the package Vegan from R (Oksanen *et al.* 2015) using the Bray-Curtis distance. Including a representation of the entire community composition of bacteria in our models is needed to clarify the relative importance of bacterial composition and diversity in driving multifunctionality in the Field survey (i.e., real world) where multiple bacterial species co-exist together.

In addition to these analyses, for the Field survey we repeated our model including richness and composition of bacteria, spatial variables (latitude and longitude) and soil properties (soil carbon and pH). Finally, for the Field survey, we also repeated our analyses including spatial influence, soil properties, bacterial richness, and composition at the OTU level (using the axes from a NMDS) instead of only including selected microbial taxa in this study (α-, β-, γ-Proteobacteria, Actinobacteria, Bacteroidetes and Fimicutes).

We ranked all the models that could be generated with our independent variables according to the second-order Akaike information criterion (AICc). Here, we consider a ΔAICc > 2 threshold to differentiate between two substantially different models and then select the best of those models (Burnham and Anderson 2002; Burnham *et al.* 2011). Then, we compared the AICc of the best model including both taxa richness and composition to that of the corresponding model with only composition or richness. Differences < 2 in AICc between alternative models indicate that they are approximately equivalent in explanatory power (Burnham and Anderson 2002). Finally, we calculated the relative importance of bacterial richness and composition (relative abundance of six selected taxa) as predictors of multifunctionality as the sum of the Akaike weights of all models that included the predictor of interest, taking into account the number of models in which each predictor appears (Burnham and Anderson, 2002; Maestre *et al.* 2012). It is important to note that, in general, our analyses were not influenced by high collinearity between richness and composition, as only weak relationships were found between bacterial richness and composition for both Field and Microcosm studies (Table S3).

Partial correlation

We conducted partial correlation analyses to thoroughly check whether the relationship between bacterial richness or composition was still maintained after controlling for the rest of microbial attributed selected in the best model.

Random Forest

To further clarify the relative importance of bacterial richness and composition in predicting multifunctionality, we conducted a classification Random Forest analysis (Breiman 2001), as done in Delgado-Baquerizo et al. (2015). Random Forest analysis for the field study includes as predictors: bacterial richness, composition and total abundance, as well as latitude, longitude, soil carbon and pH. Random Forest analyses for the experimental soils A and B include as predictors: bacterial richness, composition and total abundance. This technique is a novel machine-learning algorithm that extends standard classification and regression tree (CART) methods by creating a collection of classification trees with binary divisions. Unlike traditional CART analyses, the fit of each tree is assessed using randomly selected cases (1/3 of the data), which are withheld during its construction (out-of-bag or OOB cases). The importance of each predictor variable is determined by evaluating the decrease in prediction accuracy (i.e. increase in the mean square error between observations and OOB predictions) when the data for that predictor is randomly permuted. This decrease is averaged over all trees to produce the final measure of importance. These analyses were conducted using the rfPermute package (Archer et al. 2016) of the R statistical software (http://cran.r-project.org/).

Results

Field survey

Our distance-based multi-modeling approach indicated that bacterial richness and composition (relative abundance of β-Proteobacteria, γ-Proteobacteria, Bacteroidetes and Actinobacteria) provided independent and complementary information to predict multifunctionality (Table 1). The best-fitting model accounted for over 60% of the variation in multifunctionality; and always included both bacterial richness and composition as predictor variables (Table 1). Model fit declined substantially when we removed either bacterial richness or composition as a predictor variable (Table 1; ΔAICc >2 threshold), suggesting that both microbial components are important predictors of ecosystem multifunctionality. Specifically, the same models with composition but without bacterial richness had a significantly but modestly higher AICc than the best models including taxa richness and composition (ΔAICc of +2.23). Models including only bacterial richness had a markedly higher ΔAICc (+23.11) than the best-fitting model (Table 1). We then calculated the relative importance of all microbial attributes in predicting multifunctionality using weighted information from all models. Bacterial richness was the fourth most important predictor of multifunctionality after the relative abundance of Actinobacteria, Gammaproteobacteria and Bacteroidetes (Fig. 1).

Our results remained unchanged even when we additionally included spatial (latitude and longitude) and soil properties (soil carbon and pH; Table S5). Most importantly, our main result, that bacterial richness and composition perform independently to drive multifunctionality, was maintained after including in our model spatial influence, soil properties, bacterial richness, and bacterial composition at the OTU level (three axes of a non-metric multidimensional scaling analysis [NMDS])

(Table S6). Note that the 3D solution of this NMDS had a very low stress (0.05) indicating that the three axes of our NMDS were a good representation of the entire soil bacterial community in our Field survey. Random Forest analyses provided further evidence that bacterial richness and composition were significant predictors of multifunctionality after accounting for multiple multifunctionality drivers. Soil C and pH were the major predictors of multifunctionality followed by microbial composition and richness (Fig. S3).

Bacterial richness was positively related to multifunctionality (Fig. 2a), a result which remains consistent after controlling for latitude and longitude (Table S7), total bacterial abundance (Table S8) and the relative abundance of selected taxa in the best model (Table S9). These results were also maintained when we explored the relationship between bacterial richness and the number of functions at or above 25, 50 and 75% thresholds of the maximum observed function (Table S10). Moreover, we found positive effects of bacterial richness on some individual functions (enzyme activities and carbon degradation assays; Tables 2 and S11). For example, we found positive correlations (Spearman) between bacterial richness and β-glucosidase (P=0.01), N-Acetylglucosaminidase (P=0.08) and SIR Glucose (P<0.01) (Tables 2 and S11). Similar results were obtained when we evaluated the linear relationships among bacterial richness and single functions, with cellobiosidase, but not N-Acetylglucosaminidase, being positively related to bacterial richness in these analyses (Fig. S4).

Together, the selected bacterial phyla/classes Actinobacteria, Bacteroidetes, α-, β-, γ-Proteobacteria and Fimicutes accounted for 28-74% (average 53%) of the relative abundance of bacteria from all sites. The relative abundance of Actinobacteria, Bacteroidetes, β-Proteobacteria and γ-Protobacteria were positively related to multifunctionality (Table 3). Regarding single functions, γ-Proteobacteria was strongly related to phosphatase activity and basal respiration (Tables 2 and S11). Conversely, Bacteroidetes, β-Proteobacteria and γ-Proteobacteria were positively related to most soil functions in our Field survey.

Microcosm study (Experimental approach)

Supporting the results from our Field survey, our distance-based multi-modeling approach indicated that bacterial richness and composition (relative abundance of Bacteroidetes and Actinobacteria for Soil A and γ-Proteobacteria for Soil B) provided independent and complementary information to predict multifunctionality (Table 1). The best-fitting models accounted for significant but modest (8% for soil A) and substantial (43% for Soil B) percentages of the variation in multifunctionality for the two soils; and always included both bacterial richness and composition as predictor variables (Table 1). Also, similar to the results found for our Field survey, model fit declined substantially when we removed either bacterial richness or composition as a predictor variable (Table 1; ΔAICc > 2 threshold), providing evidence that both microbial components are important predictors of ecosystem multifunctionality. Specifically, the same models with composition but without bacterial richness had a higher AICc than the best models including taxa richness and composition for Soil A (+26.69) and Soil B (+12.84). Similarly, models including only bacterial richness had a higher ΔAICc for Soil A (+67.35) and Soil B (+3.10). Mean values for multifunctionality in each of the 68 experimental microbial combinations for soils A and B are available in Fig. S5.

Although models including both bacterial richness and composition always improved multifunctionality predictions (vs. those models lacking one of these components; Table 1), our results for the Microcosm study also suggested that the relative importance of bacterial richness compared with composition is soil-dependent. Thus, richness was more important than composition in Soil B, while the opposite pattern was observed for Soil A (Table 1). Similar results are found when we calculated the relative importance of bacterial richness and composition using weighted information from all models (Fig. 1). Alternatively, our Random Forest model indicated that bacterial richness was the most important predictor of multifunctionality, but only after the relative abundance of Actinobacteria for soil A and Gammaproteobacteria for soil B (Fig. S3).

Moreover, our Microcosm study provided evidence that the identity of the most relevant microbial taxa is also soil-dependent. Thus, while Bacteroidetes and Actinobacteria were the strongest predictors for Soil A (Table 1; Fig. 1), γ-Proteobacteria was the main predictor of multifunctionality in Soil B (Table 1; Fig. 1). Interestingly, observational data from these two samples were consistent with what we observed in our Microcosm study. Thus, the models based on the Field survey included the main bacterial taxa in both soils from the Microcosm study and included β-Proteobacteria, γ-Proteobacteria, Bacteroidetes and Actinobacteria in the best models (Table 1).

We found the highest multifunctionality in the soil microcosms with the highest bacterial richness in both Soils A and B (Figs. 2b and c; $P < 0.01$). These results remained consistent after statistically controlling for total bacterial abundance (Tables S8 and S12; Fig. S6). In addition, the positive effect of bacterial richness on multifunctionality was maintained after we removed key taxa from the analyses, demonstrating that this effect was not just due to key taxa (sampling effect) (Fig. S7). These results were also maintained when we explored the relationship between bacterial richness and the number of functions at or above 25, 50 and 75% thresholds of the maximum observed function

(Table S10) and also after controlling for the relative abundance of selected taxa in the best model (Table S9). For single functions, bacterial richness was positively related to N-Acetylglucosaminidase and phosphatase activities in both soils from our Microcosm study and to β-glucosidase and cellobiosidase activities in Soil A ($P < 0.05$; Tables 2 and S11 and Figs. S8 and S9).

In the Microcosm study, bacterial composition effects on multifunctionality were soil dependent (see Fig. S10 for original bacterial composition in "soils A and B"), with a positive correlation between multifunctionality and Actinobacteria and Bacteroidetes and a negative correlation with β-Proteobacteria in soil A (Table 3), while in Soil B there was a positive correlation of multifunctionality and γ-Proteobacteria. Similar results were found when we explored the relationship between bacterial composition and the number of functions at or above 25, 50 and 75% thresholds of the maximum observed function (Table S10). Consistent with these findings, the highest multifunctionality in the diverse communities and the monocultures (i.e. bacterial taxa identity effects based on presence/absence analyses) was found for Actinobacteria and Bacteroidetes in Soil A and Proteobacteria classes in Soil B (Fig. 3; $P < 0.01$).

Moreover, the effects of bacterial composition on individual functions were also soil dependent (Tables 2 and S11). For example, γ-Proteobacteria, which was strongly related to phosphatase activity and basal respiration in Field survey (Tables 2 and S11), had a predominant positive effect on soil functions from Soil B including N-Acetylglucosaminidase, basal respiration and SIR glucose and lignin (Tables 2 and S11). Conversely, Actinobacteria and Bacteroidetes, which were positively related to a wide array of functions in the Field survey, showed predominantly positive effects on functions in Soil A including β-glucosidase, cellobiosidase and N-Acetylglucosaminidase, but also phosphatase and SIR Lignin in particular case of the isolated bacteria from the phylum Actinobacteria (Tables 2 and S11).

Discussion

Despite the growing body of literature providing evidence that microbial diversity promotes ecosystem functioning in terrestrial ecosystems (Jing *et al.* 2015; Delgado-Baquerizo *et al.* 2016), most studies have tended to focus on a particular component of diversity (richness or composition), and no previous study, to the best of our knowledge, has empirically and statistically examined the relative importance of both bacterial richness and composition in supporting multiple functions in terrestrial ecosystems. Using observational and experimental data, we provide evidence that both bacterial richness and composition are key drivers of multiple ecosystems functions in terrestrial ecosystems. Most importantly, our multi-model approach indicates that these two microbial diversity components provide independent and complementary information on the role of bacteria in ecosystem processes. These results provide strong support for the hypothesis that the effects of bacterial biodiversity on ecosystem functioning are due to the combined effects of bacterial richness and identity of key taxa within a community. Ours is, to our knowledge, the first attempt to evaluate the relative importance of both diversity and composition of bacteria, the most diverse and abundant organisms on Earth, in driving multifunctionality. However, future studies exploring the relative importance of microbial drivers of multifunctionality should be encouraged to include diversity and composition of fungi to obtain a broader picture of the role of microbial diversity and composition in driving multifunctionality.

Both our field survey and microcosm study provide evidence that bacterial richness is strongly positively related to multifunctionality. Our results were maintained after controlling for spatial structure (in observational data) and microbial abundance using partial correlations and ANCOVA analyses, and provided experimental support to previous observational studies showing positive relationships between soil microbial diversity and multiple soil functions, such as those used here (He *et al.* 2009; Jing *et al.* 2015; Delgado-Baquerizo *et al.* 2016). The mechanisms behind the positive effects of bacterial richness on multifunctionality could include an increase in the interactions among microbial taxa (complementarity effects; Loreau & Hector 2001) and the so-called “sampling effect” (i.e. increasing taxa richness increases the likelihood that key taxa would be present; Hooper *et al.* 2005). Species interactions are especially important for microbial communities that rely heavily on aggregated processes (Schimel *et al.* 2005) such as organic matter decomposition as an energy source. These aggregated processes involve many metabolic routes and require the cooperation of large and diverse groups of microbes to break down complex and recalcitrant polymers into simpler, more labile monomers, which are rapidly consumed and largely respired (Schimel *et al.* 2005). Thus, losses in bacterial richness may inactivate critical functions (e.g. chitin degradation), but also can reduce the rates in which multiple ecosystems functions are being produced, as supported by our observational and experimental data. Bacterial richness also showed similar positive trends with each of the single functions studied. Of particular interest was the fact that bacterial richness showed a strong and positive effect on N-Acetylglucosaminidase (chitinase) in all the experimental approaches used here. Chitin is an extremely complex compound, is a structural component of many organisms, and is widespread in nature (Beier & Bertilsson 2013). Bacteria are believed to be major mediators of chitin degradation, a complex process that involves several metabolic reactions with important implications for carbon and nitrogen cycling (Beier & Bertilsson 2013). This result further supports the notion that complex processes such as organic matter decomposition are favored by the existence of a diverse collection of microbes all contributing to the overall process to promote the highest degradation rates (Schimel *et al.* 2005). Our Microcosm study also showed that a "sampling effect" may be, at least in part, responsible for driving multifunctionality, as microcosms including certain key taxa tended to have the greatest multifunctionality. Interestingly, bacterial richness had a positive effect on multifunctionality even after the effects of key species were accounted for (removing them) in our analyses (Fig. S6; Appendix S1). Consistent with the results reported by Hooper *et al.* (2005) for plant communities, we suggest that microbial taxa interaction and sampling effects are not mutually exclusive.

Bacterial composition was also a strong predictor of multifunctionality in both Field and Microcosm studies. However, unlike bacterial richness, the effects of bacterial composition on multifunctionality varied with both soil properties and ecological characteristics of the specific bacterial taxa, especially under the Microcosm study. For example, for Soil B (high soil carbon), γ-Proteobacteria enhanced multifunctionality in both single and mixed cultures. Similarly, γ-Proteobacteria, which was positively related to soil carbon (Table S13), also showed a positive relationship with multifunctionality across the Field survey. Class γ-Proteobacteria tend to exhibit copiotrophic life histories (Fierer *et al.* 2007; Trivedi *et al.* 2013), preferring environments that are rich in carbon, promoting the greatest multifunctionality and supporting critical processes such as complex and labile carbon decomposition. Thus, this Proteobacteria class may be critical for supporting multifunctionality in carbon-rich soils. Conversely, for Soil A (lowest soil carbon) in the Microcosm study, we found a predominant effect of Actinobacteria in supporting multifunctionality. Actinobacteria are defined as oligotrophs (Bastian *et al.* 2009; Trivedi *et al.* 2013), and are more competitive in soils with low levels of carbon such as those from drylands (Maestre *et al.* 2015). In Soil A, Actinobacteria was also strongly positively related to extracellular enzyme activity and lignin degradation content. Our findings are supported by the results of previous studies suggesting that Actinobacteria contains a broad array of genes that allow the breakdown and utilization of recalcitrant organic compounds such as lignin, chitin and cellulose that can be used under stressful soil conditions (low carbon; Bastian *et al.* 2009; Trivedi *et al.* 2013).

Interestingly, only the relative abundance of Bacteroidetes was consistently selected as a major predictor of multifunctionality in all experimental approaches and statistical models used here. In general, the relative abundance of Bacteroidetes, defined as copiotrophic organism by Fierer *et al.*

(2007), promoted high rates of multifunctionality, enzyme activities and/or respiration rates in all experimental approaches (Table 2). More specifically, the relative abundance of Bacteroidetes always promoted the activity of chitin degradation in all soils. These results are in agreement with a previous study highlighting their potential to break down chitin and cellulose in terrestrial ecosystems (Trivedi *et al.* 2013); and further highlight the importance of this taxa in regulating organic matter decomposition and C cycling in soil.

An important finding of our study is that although both components of biodiversity are important drivers of multiple ecosystem processes related to organic matter decomposition and nutrient cycling, the relative importance of richness compared with composition in controlling multifunctionality is soil-dependent, as supported by our Microcosm study (Soil A vs. B). In particular, we found that richness is more important than composition in Soil B, with the higher soil organic matter, while the opposite pattern occurred in Soil A. Although we cannot extrapolate from only two soils, if these results were generally true, they would suggest that bacterial richness might play a predominant role in organic soils, where the interaction among multiple microbial communities is needed to break down complex and recalcitrant polymers into simpler and more labile monomers (organic matter degradation; Schimel *et al.* 2005). Conversely, species identity (Bacteroidetes and Actinobacteria in Soil A) may play a major role in mineral soils. For instance, Actinobacteria have been shown to possess important adaptations that enable them to resist environmental harshness (ability to survive desiccation and low nutrient availability conditions; Battistuzzi & Hedges 2009). Thus, these results support the notion that both microbial richness and composition are needed to accurately estimate the consequences of losses in microbial diversity (from global environmental changes such as climate change and land use intensification) on ecosystem functioning.

Interestingly, observational data were consistent with what we observed in our Microcosm study, providing insights into the main microbial pattern controlling multifunctionality in terrestrial ecosystems, and demonstrating the value of using each of these approaches. For example, in both the field and microcosm studies, an increase in taxa richness was positively related to multifunctionality. Of particular novelty, the Field data provided a comprehensive view of the main taxa controlling multifunctionality in Soils A and B and suggest that Actinobacteria, Bacteroidetes and γ-Proteobacteria are the main drivers of multifunctionality in terrestrial ecosystems at a large scale. All of these bacterial taxa are globally distributed and dominant in many terrestrial ecosystems worldwide (Fierer *et al.* 2009; Maestre *et al.* 2015). This result suggests that observational data can be useful for predicting microbial community shifts and their consequences for ecosystem functioning under global change, but also that this observational data will be useful in developing generic algorithms to be included in global biogeochemical models.

In conclusion, our findings provide strong evidence, from two independent approaches, that bacterial richness and composition are important, yet independent drivers of multiple ecosystem functions related to organic matter decomposition and nutrient cycling. Greater microbial richness and globally-dominant bacterial taxa such as γ-Proteobacteria, Actinobacteria and Bacteroidetes were critical drivers of multifunctionality in both our field and microcosm studies. Information on both microbial richness and composition therefore need to be considered when formulating sustainable management and conservation policies, and when predicting the effects of global change on ecosystem functions. These findings advance our understanding of the mechanisms underpinning relationships between biodiversity and ecosystem functionality in terrestrial ecosystems, and reinforce the need to develop approaches and policies to protect soil microbial diversity and their positive effects for multiple ecosystems functions.

Conflict of Interest

The authors declare no competing financial interests.

Author´s contributions

M.D-B. conceived the idea of this study and designed experiments in consultation with B.K.S., P.B.R. and P.T. Soil sampling was conducted by M.D-B. and D.J.E. Laboratory analyses were done by M.D-B., P.T. and C.T. Bioinformatics analyses were done by T.C.J. Statistical modeling was conducted by M.D-B. The manuscript was written by M.D-B with contributions from all co-authors.

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Data accessibility.

The primary data used in this study is available in the Dryad Digital Repository. http://dx.doi.org/10.5061/dryad.h5q34

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Figure legends

Figure 1. Relative importance of bacterial richness and composition in models of multifunctionality for the field (a) and experimental studies (b-c). The height of each bar is the sum of the Akaike weights of all models that included the predictor of interest, taking into account the number of models in which each predictor appears.

Figure 2. Effects of bacterial richness on multifunctionality for Field (a) and Microcosm (b and c) studies. Bacterial diversity in Field survey is calculated as the number of OTUs (97% similarity; x^2-transformed). Bacterial diversity in the Microcosm study ("soil A and B") is the number of bacterial phyla/classes. The solid lines in figure a represents the fitted linear regression. Data in Fig b (Soil A) and c (Soil B) represent means ± SE. Different letters in panels b) and c) indicate significant differences between richness levels (P<005) in multifunctionality index (*post-hoc* tests after one-way ANOVA).

Figure 3. Mean (±SE) values for multifunctionality across different bacterial taxa for mono- (a and c) and mixed cultures (b and d) of bacteria in the experimental approach. Different letters in panels a) and c) indicate significant differences in multifunctionality among bacterial taxa (*P* < 0.05) as tested using post-hoc tests after one-way ANOVA. Panels b) and d) represent averaging multifunctionality index in mixed cultures including (presence) or excluding (ausence) each bacterial phylum/class. In these panels significance levels are as follows: ** *P* < 0.01, *** *P* < 0.001.

Table 1. Best-fitting model (including microbial richness and composition) and the same model with either bacterial richness or composition (but not both) included as predictors of multifunctionality for the Field and Microcosm ("soils A and B") studies. Shaded cells indicate that the variable has been included in the model. Models are ranked by AICc. AICc measures the relative goodness of fit of a given model; the lower its value, the more likely the model to be correct. ΔAICc is the difference between the AICc of each model and that of the best model. ΔAICc indicates substantially different models. A = α-Proteobacteria; B = β-Proteobacteria; C = γ-Proteobacteria; D = Firmicutes; E = Bacteroidetes; F = Actinobacteria.

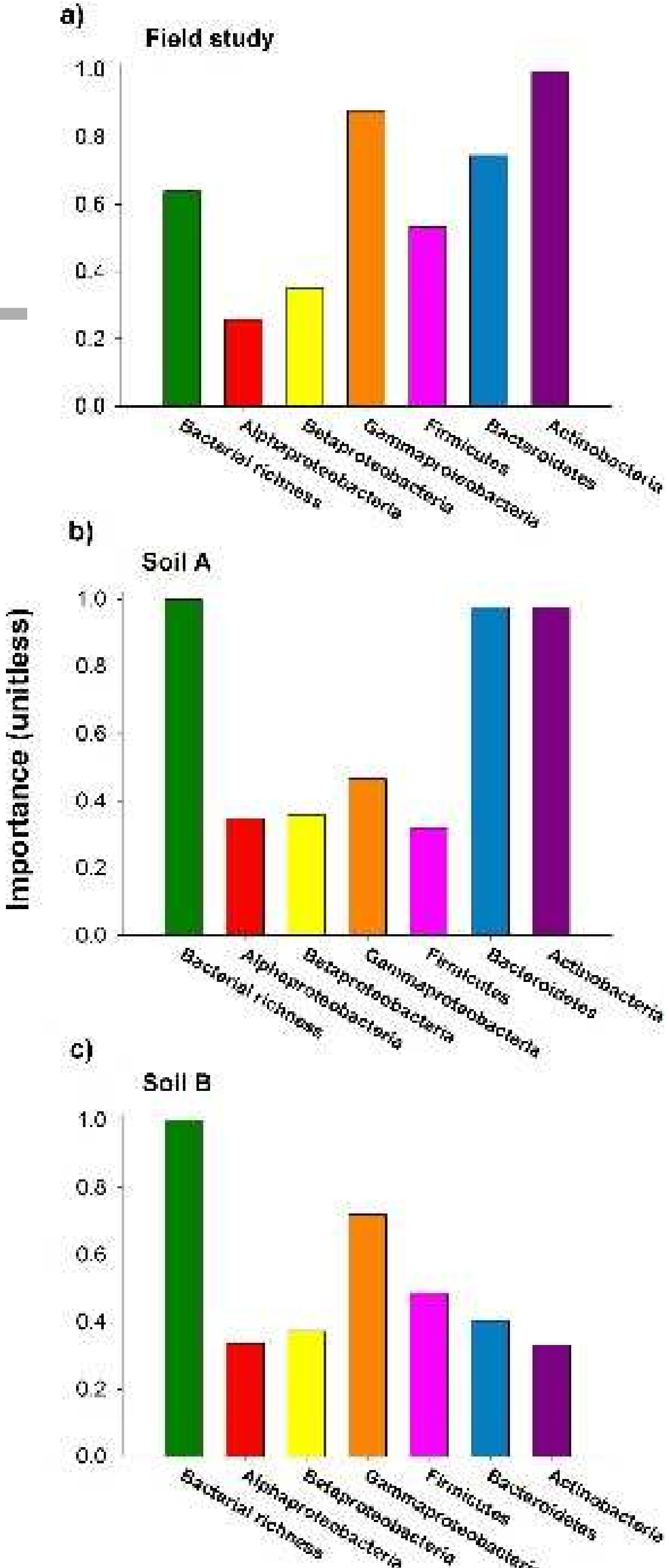
Approach	Diversity	Composition	R^2	AICc	ΔAICc
I (Field study)	Richness	γ-Proteobacteria + Firmicutes + Bacteroidetes+ Actinobacteria	0.599	-53.75	0.00
	Excluded	γ-Proteobacteria + Firmicutes + Bacteroidetes+ Actinobacteria	0.551	-51.52	2.23
	Richness	Excluded	0.134	-30.64	23.11
II (Soil A)	Richness	Bacteroidetes+ Actinobacteria	0.429	-445.74	0.00
	Excluded	Bacteroidetes+ Actinobacteria	0.344	-419.05	26.69
	Richness	Excluded	0.190	-378.39	67.35
II (Soil B)	Richness	γ-Proteobacteria	0.084	-276.88	0.00
	Excluded	γ-Proteobacteria	0.014	-264.04	12.84

Table 2. Summary of the effects of microbial composition on the multiple ecosystems functions in this study for the field and microcosm (soils A and B) studies. Microbial composition effects are based on Spearman correlations available in Table S7. Symbols + and - indicate positive and negative interactions.

Study	Functions	Richness	α-Proteobacteria	β-Proteobacteria	γ-Proteobacteria	Firmicutes	Bacteroidetes	Actinobacteria
Field	**β-Glucosidase**	+		+	+		+	+
	Cellobiosidase			+	+		+	
	N-Acetylglucosaminidase	+		+	+		+	
	Phosphatase		+	+	+			
	Basal Respiration		+	+	+		+	+
	SIR Glucose	+		+			+	+
	SIR Lignin			+	+		+	
Microcosm (Soil A)	**β-Glucosidase**	+		-	-		+	+
	Cellobiosidase	+					+	+
	N-Acetylglucosaminidase	+		-	-		+	+
	Phosphatase	+						+
	Basal Respiration	-		-	+			
	SIR Glucose			+				
	SIR Lignin		-		-	+		+
Microcosm (Soil B)	**β-Glucosidase**							
	Cellobiosidase							
	N-Acetylglucosaminidase	+			+	+	+	
	Phosphatase	+						+
	Basal Respiration			-	+	-		
	SIR Glucose				+	-		
					🟩	🟥		

Table 3. Correlations (Spearman) between main bacteria taxa and multifunctionality for field and M microcosm (soils A and B) studies (n = 204). P-values are in brackets.

Study	α-Proteobacteria	β-Proteobacteria	γ-Proteobacteria	Firmicutes	Bacteroidetes	Actinobacteria
Field	0.111 (0.480)	0.570 (<0.001)	0.566 (<0.001)	-0.007 (0.963)	0.637 (<0.001)	0.291 (0.058)
Microcosm (Soil A)	-0.036 (0.612)	-0.153 (0.029)	-0.108 (0.124)	-0.080 (0.257)	0.297 (<0.001)	0.532 (<0.001)
Microcosm (Soil B)	-0.003 (0.964)	0.074 (0.294)	0.223 (0.001)	-0.050 (0.474)	0.021 (0.764)	0.082 (0.244)



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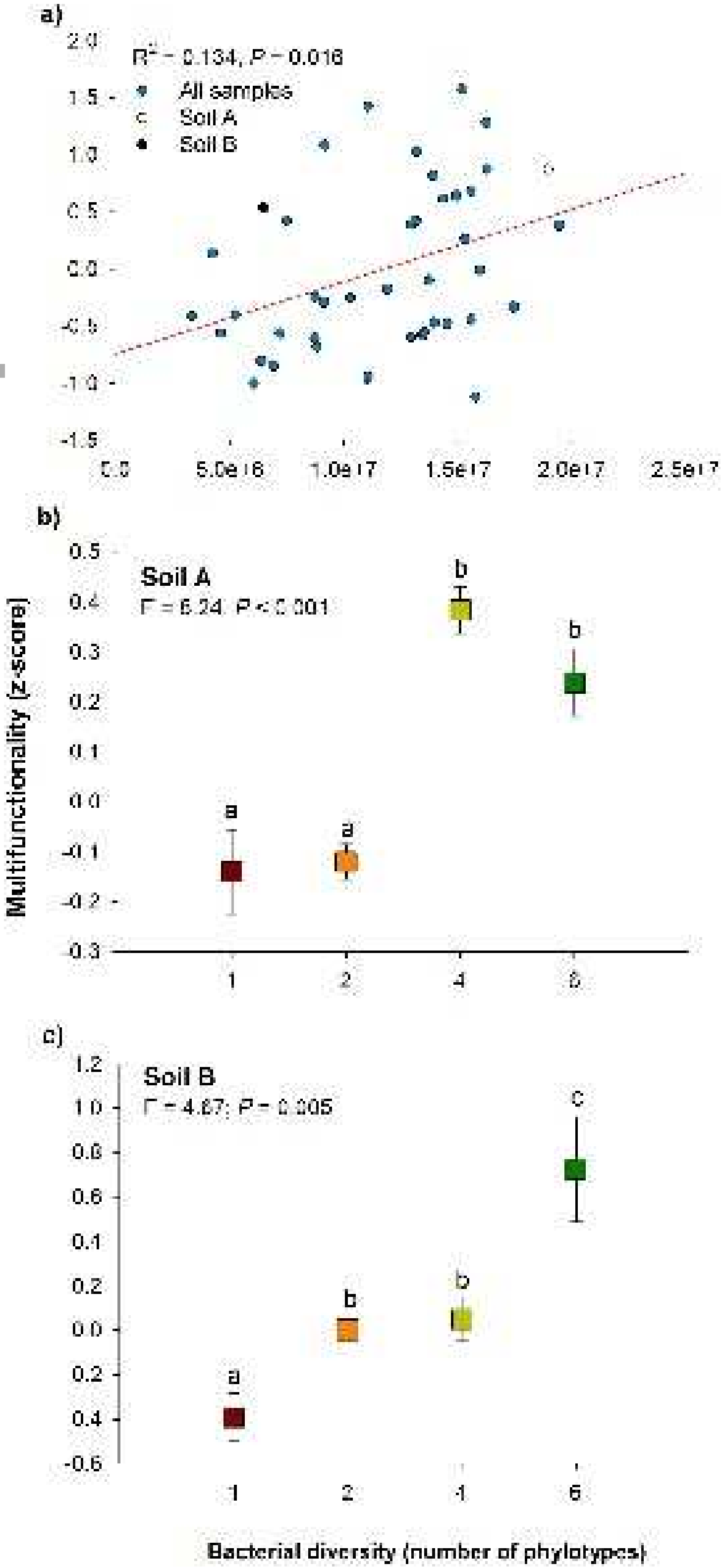

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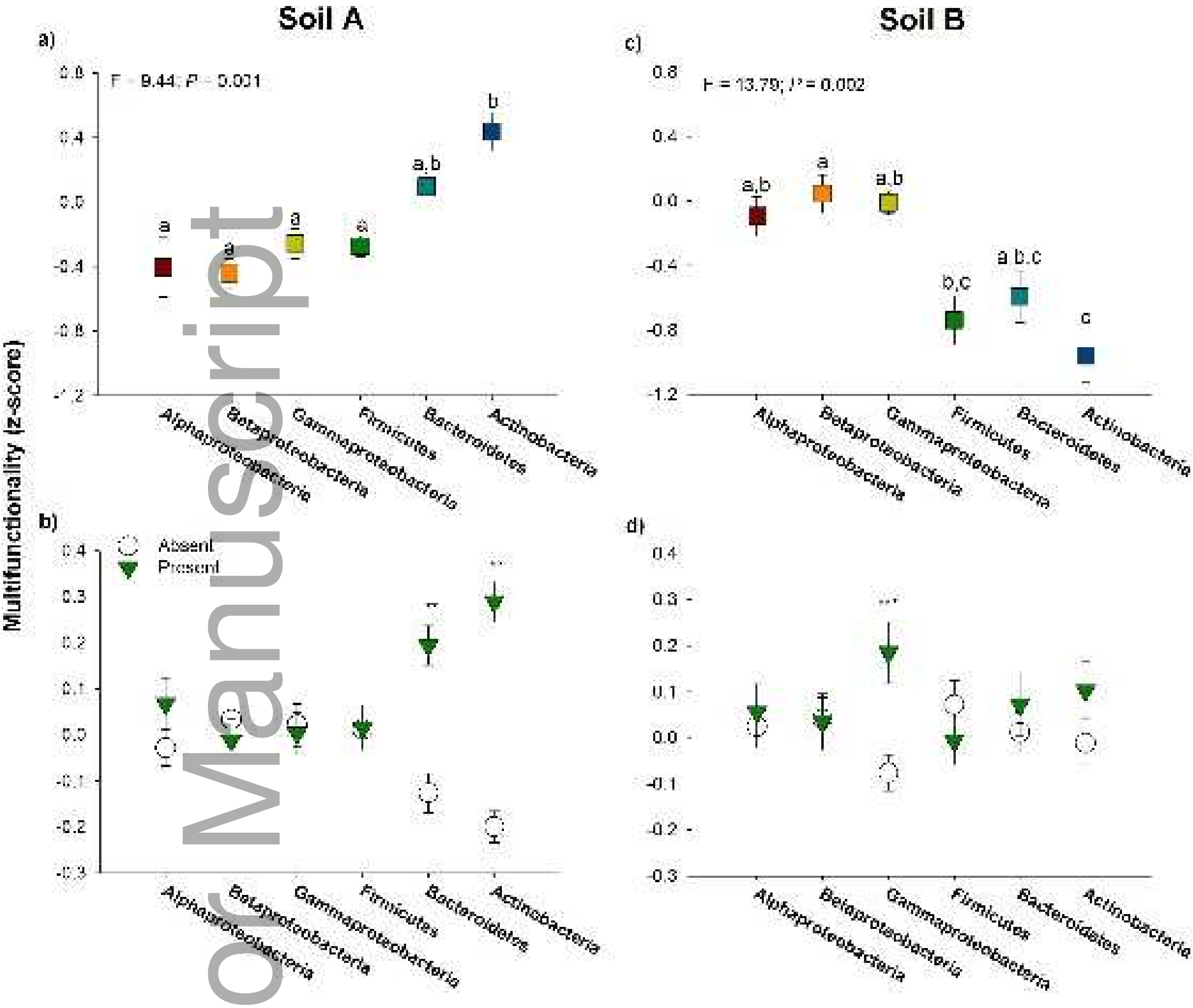

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