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Abstract

The core microbiota defines the fraction of microorganisms common to all individuals from the same host species regardless of the abiotic context, be they located inside (e.g. animal guts) or outside (e.g. plant rhizospheres). While the core microbiota of many host species have been documented, no studies attempted to decipher how these core microbiota could be altered when their respective host species are interacting. We thus tested the hypothesis that interactions between different host species possessing external microbiota could result in a novel emerging entity: a core microbiota of an interaction. This is particularly true in soils, where such interactions are likely to occur between different host species harboring external microbiota, like plants through rhizospheres and earthworms through drilospheres. Using three contrasting soils (sand, loam or clay) and a meticulous sampling of different microhabitats (rhizospheres, casts and bulk) coupled to a "source-sink approach" derived from the meta-community theory, we evidenced the presence of a conserved core microbiota of bacterial OTUs resulting from plant-earthworm interactions in all soils. This interaction was also evidenced using a tailored network analysis, revealing the presence of signature OTUs always found in earthworms casts and plant rhizospheres, and whose co-occurrence patterns were indicative of soil type. Furthermore, qPCR abundance estimates revealed that not only bacteria, but also fungi and archeae, are affected by plant-earthworm interactions. Our findings provide a new framework to explore aboveground-belowground interactions through the prism of microbial communities.

1. Introduction

Microorganisms participate in crucial soil functions and services like biogeochemical cycles, bioremediation and food production. Their activities strongly depend on soil ecosystem engineers (Jones *et al.* 1994; Lavelle *et al.* 1997) which "directly or indirectly modulate the availability of resources to other species, by causing physical state changes in biotic or abiotic materials". As a consequence, soil is made of "microbial hotspots" (Kuzyakov & Blagodatskaya 2015), previously described as specific "functional domains", defined as "parts of the soil that are influenced by a major biotic or abiotic regulator, […] recognizable in a set of structures (pores, aggregates, fabrics etc.) generated by the regulator that can be physically separated from the soil matrix" (Lavelle, 2002). In the case of plants and earthworms, two major soil ecosystem engineers, these functional domains are respectively named the rhizosphere and drilosphere, which are also microhabitats for the myriads of microorganisms populating them, being the microbiota. In this study, not only we investigated how plants and earthworms do influence soil microbial communities in their respective functional domains, but also how their interaction can reciprocally alter the microbiota residing in these two microhabitats.

By definition, the rhizosphere includes soil aggregates that are closely adhering to roots, and the drilosphere encompasses two soil parts that have been modified by earthworms, namely the casts (earthworms excreta) and the burrows (the inner part of earthworms galleries) (Lavelle, 2002). Microorganisms populating these two microhabitats could either come from the surrounding bulk soil matrix and/or from the host itself (e.g. endophytes and earthworm's cuticle/gut). While the influence of plants on soil microbial communities is well documented (e.g. Philippot *et al.*, 2013), the overlooked role of earthworms may be as important (Brown *et al.* 2004; de Menezes *et al.* 2018; Blouin *et al.* 2019; Medina-Sauza *et al.* 2019), given they represent the largest soil animal biomass in most terrestrial ecosystems (Bouché 1972). Indeed, root microbiota are renowned for their importance on plant health (Tkacz & Poole 2015; Pérez-Jaramillo *et al.* 2016; Finkel *et al.* 2017), but so far very few is known regarding the role of microbes leaving in contact with earthworms

(Medina-Sauza *et al.* 2019). Moreover, effects of plants and earthworms on soil microbial communities are often studied independently. To our knowledge, there are no studies on the effect of plants on microbial communities in the drilosphere and only one report on earthworm's effect on rhizosphere microbial communities (Braga *et al.* 2016). Considering their respective capacity to manipulate microbes, one could regard plants and earthworms as competitors for the steering of soil microbial communities and functions. However, a positive interaction between the two is generally observed, as several studies showed that earthworms can increase plant growth (van Groenigen *et al.* 2014; Blouin *et al.* 2019), with converging observations suggesting that plant-earthworm relationships are partly mediated by microbes (Puga-Freitas *et al.* 2012a/b; Blouin 2018).

Going further, no attempt has been made to determine if these reciprocal influences remains despite variation in the soil physico-chemical context. This question is in line with the concept of "core microbiota", defined as the faithful fraction of microorganisms always associated to their host species individuals regardless of environmental fluctuations, and characterized through taxonomical genetic markers (Turnbaugh *et al.* 2009). It was illustrated in many instances, such as the Human gut (Turnbaugh *et al.* 2009), insect gut (Shukla *et al.* 2018) and plant rhizospheres (e.g. *Arabidopsis thaliana*, Lundberg *et al.* 2012; *Lactuca sativa L.*, Chowdhury *et al.* 2019). For humans or animals, the core microbiota is generally accessed *via* feces, a proxy to study the gut without harm (Vandeputte *et al.* 2017). For plants, the core microbiota is generally investigated in the rhizosphere, which is often referred to as an "inverted" gut system (Ramírez-Puebla *et al.* 2013).

As both rhizosphere and drilosphere microbiota are external to their respective hosts, located into soil, an interaction between these two microbial communities could occur. We investigated this interaction in three different soils (predominance of sand, loam or clay) using microcosms containing either none, one or two soil engineer species as follow: i) a plant (barley, *Hordeum vulgare*), ii) endogeic earthworms (*Aporrectodea caliginosa*), iii) both macroorganisms and iv) a control without macroorganisms (Fig.1). Looking at the different scales through the prism of microhabitats, we expected to capture the reciprocal influences of plants on cast microbiota (used as

a proxy for drilosphere), and earthworms on rhizosphere microbiota. Thus, we applied a meticulous soil dismantling to separately collect i) the bulk soil either from the treatment without macroorganisms (control bulk) or near rhizospheres and casts (plant bulk, earthworm bulk, plant-earthworm bulk), ii) rhizospheres (with/without earthworms) and iii) casts (with/without plants; Fig.1). We made the following hypotheses for the presence of i) core microbiota for *Aporrectodea caliginosa* and *Hordeum vulgare* respectively found in casts and rhizospheres; but also ii) a core microbiota of the plant-earthworm interaction simultaneously found in both rhizospheres and casts; as well as iii) a core microbial network common and specific of both rhizospheres and casts. Together with plant traits, we analyzed the molecular abundance of microbes (bacteria, archaea, and fungi) and focused on the bacterial community structure using high throughput sequencing to seek, for the first time, the existence of a core microbiota of an interaction between two host species.

2. Materials and methods

2.1 Experimental design and sampling

Three soils were used (Tab.1): a sand soil (cambisoil with moor from CEREEP station, Saint-Pierre-Lès-Nemours, France), a loam crop soil (luvisoil from INRA, Versailles, France) and a forest clay soil (leptosoil from MNHN station, Brunoy, France). The first 20cm were sampled excluding plant material. Soils were air-dried, sieved (2mm) and set in microcosm pots of 1l containing 1kg of soil watered at 80% of their respective water holding capacity, being the optimum for plant and earthworm (Lavelle 1978). Four conditions were tested with five biological replicates (Fig.1): i) a plant, ii) three earthworms, iii) both together and iv) nothing (control). Barley (*Hordeum vulgare* L. commercial variety, “La fermette”) was germinated in three batches of 80 seeds in Petri dishes containing humidified soil (100%, 20°C, phytotron, seven days). ~8cm-tall seedlings were transplanted in pots. The earthworm *Aporrectodea caliginosa* was chosen for its endogeic lifestyle. Individuals originated from a non-stop breeding program initiated in 2007 (IRD park, Bondy, France). Three batches of young individuals were purged in their respective experimental soil to prevent breeding substrate contaminations (three days). Three individuals were

introduced at the pot surfaces (total weight ~1g). Microcosms were incubated in a climatic chamber (S10H, Conviron, Canada; 75% air humidity, 18/20°C night/day, 12h photoperiod with constant 300 µmol photons m-2 s-1, 28 days). Leaf surface was estimated after 17 days by summing leaf areas (leaf area = leaf length x mid-section leaf width x 0.75, Blouin *et al.* 2007). Plant height was estimated after 23 days based on the longest leaf. After 28 days, all microcosms were used for destructive sampling. Soil was meticulously sampled to recover the distinct microhabitats: rhizospheric soil (adhering root soil recovered from vigorous shaking with distillated water, then centrifuged), earthworm casts (visual identification; Velasquez *et al.* 2007) and bulk soil (no visible macroorganisms influence). Shoot biomass was measured after drying (50°C, 48h).

2.2 qPCR settings

Total DNA was extracted from 250 mg of soil using FastDNA® SPIN Kit for Soil (MP Biomedicals). DNA concentration was quantified using Quant-iT™ dsDNA High-Sensitivity Assay Kit (Invitrogen) before dilution at 1 ng.µl-1. Fungal ITS (ITS3F: 5'-GCATCGATGAAGAACGCAGC-3', ITS4R: 5'-TCCTCCGCTTATTGATATGC-3'; White 1990), Crenarchaeota 16S rRNA (Crenar771F: 5'-ACGGTGAGGGATGAAAGCT-3', Crenar975R: 5'- CGGCGTTGACTCCAATTG-3'; Ochsenreiter *et al.* 2003) and bacterial 16S rRNA genes (341F: 5'-CCTACGGGAGGCAGCAG-3', 534R: 5'-CCTACGGGAGGCAGCAG-3'; Muyzer *et al.* 1993) were quantified using real-time polymerase chain reactions (qPCR) on a StepOnePlus™ Real-Time PCR system (Applied Biosystem, France). Reaction mixtures (15µl) were composed of 7.5µl Power SYBR™ Green PCR Master Mix with ROX (Applied Biosystem), 1.5µl of each primer (10µM), 2.5µl UltraPure™ DNase/RNase-Free Distilled Water (Applied Biosystem) and 2µl diluted-DNA template. Potential inhibition was assessed by adding 2µl of known concentration of plasmid to reaction mixtures (adjusted water volume: 0.5µl). No inhibition was detected, as amplifications using primers targeting T7 and SP6 RNA polymerase promoters were similar amongst samples. Amplification was set with 900 s at 95 °C for enzyme activation, 35 cycles of 15 s at 95 °C, 30 s at annealing temperatures (ITS/Archaea: 55°C, Bacteria: 60°C), 30 s at

72 °C for elongation and 30 s at 80 °C for termination. Abundances were quantified with linearized plasmid-based standard curves (StepOne™ Software v2.2.2, three technical repetitions). Copy numbers were normalized per µgram of DNA per gram of soil, and log2-transformed for analysis.

2.3 16S rRNA gene amplicon sequencing

Bacteria were selected for their importance in plant-earthworm interaction (Hoeffner *et al.* 2018). Amplicons were generated from purified DNA by LGC Genomics (GmbH, Germany), respecting best practices guidelines (Berry *et al.* 2011; Schöler *et al.* 2017). The bacterial 16S rRNA gene V3-V4 hypervariable region was amplified with fusion primers U341F (5'-CCTACGGGNGGCWGCAG -3') and 785R (5'- GACTACHVGGGTATCTAAKCC -3'; Klindworth *et al.* 2013) equipped with barcode sequences for each sample, with the following protocol: 20µL reaction mixtures, 1.5 units MyTaq DNA polymerase (Bioline, Germany), 2 µl of BioStabII PCR Enhancer (Sigma, Germany), 15pmol of each primer, 5 ng template DNA, 96°C for 2 min followed by 30 cycles (96°C for 15s, 50°C for 30sec), 70°C for 90s and termination (72°C, 10 min). Amplicon were visualized (2% agarose electrophoresis, ~500 bp). Two pools of 48 samples (20 ng each) were generated (n = 96), purified once (AMPure XP beads, Agencourt, Germany) and twice (MinElute columns, Qiagen, Germany). 100 ng of purified pools were used for Illumina library construction (Ovation Rapid DR Multiplex System 1-96, NuGEN, Germany). Libraries were pooled and size-selected by electrophoresis. Sequencing was performed on MiSeq (Illumina MiSeq reagent kit v2, 2x250 bp), followed by demultiplexing and trimming of adaptors/barcodes (Illumina MiSeq Reporter software v2.5.1.3). Sequences were analyzed in-house with a Python notebook. Sequences were assembled (PEAR, default settings, Zhang *et al.* 2014), removing short sequences and applying quality checks (QIIME, Caporaso *et al.* 2010). Reference-based and *de novo* chimera detection, and Operational Taxonomic Unit (OTU, 97%) clustering were performed using VSEARCH (Rognes *et al.* 2016), and taxonomy was assigned (UCLUST, Edgar 2010) with the Greengenes database (v05, MacDonald *et al.* 2012). OTU representative sequences were aligned (PyNAST, Caporaso *et al.* 2011) to build a phylogenetic tree (FastTree,

Price *et al.* 2009). The contingency table was set at OTU level. Rarefaction curves were calculated with *vegan* (Dixon *et al.* 2009) in *Rgui* (R Development Core Team 2017) to assess sequencing depth before rarefaction (Fig.S1, rarefaction at 6900 counts).

2.4 Beta-diversity and multivariate analysis

Rarefied OTU tables and unifrac trees were used to build variance-adjusted weighted and unweighted unifrac-based analysis using distance based redundancy analysis (dbRDA, capscale, *vegan*). The rarefaction curves of raw datasets, as well as the global variance partition model is presented in supporting information (Fig.S1, Tab.S1). OTUs whose abundance was significantly altered in rhizosphere and casts when both macroorganisms were present (but not in bulk soils) were detected using the non-rarefied data with a quasi-likelihood F-test under negative binomial distributions and generalized linear models (nbGLM QLFT, FDR-adjusted $q < 0.05$), as recommended in the literature (MacMurdies & Holmes 2014; Schöler *et al.* 2017). OTUs significantly changed were extracted *via* hierarchical clustering in heatmaps for cast and rhizosphere samples (Fig.S2-Fig.S3), followed by synthetic grouping in bar charts. The grouping of cast and rhizosphere OTUs responding with a similar pattern to the presence of the other macroorganism was tested and validated with multiple Monte-Carlo simulation using null-models against the all other non-responding OTUs (Fig.S4), as described previously (Jacquiod *et al.* 2018). The list of OTUs responding in casts and rhizospheres to the presence of the other macroorganisms in summarized in supporting information (Tab.S2). A Venn diagram (*limma*, Ritchie *et al.* 2015) was done to define the core microbiota shared between casts and rhizospheres. Phylogenetic signals between OTUs identified as part of the core microbiota of the plant-earthworm interaction were assessed using weighted Mean Nearest Taxon Distance method (MNTD, package *picante*, Kembel *et al.* 2010) against a null-model in the unifrac phylogenetic tree (n = 10000 permutations, Tab.S3).

2.5 Network analysis

In order to identify the presence of a potential core network of OTUs between the rhizospheres and the casts, but not in the bulk soils, we tailored a custom methodology based on network arithmetic described in a workflow diagram in supporting data (Fig.S5). First, to account for the strong soil effect, OTU abundances in each sample were normalized into z-scores using their average and standard deviation in the control bulk soil without macroorganisms (Fig.S5, Phase 1). We focused on cosmopolitan OTUs present at least in 50% of samples in each soil (n > 16/32) and used their standardized abundances to build three correlation networks, one per microhabitat, with the *igraph* R package (Csardi & Nepusz 2006) with stringent cut-offs (Spearman's rho > |0.6|, FDR-adjusted $q < 0.05$) (Fig.S5, Phase 2). This resulted in a "bulk network" (using all standardized bulk samples in earthworm, plant, earthworm/plant treatments in the three soils, n = 36), a "cast network" (using all standardized cast samples in the three soils, n = 24), and a "rhizosphere network" (using all the standardized rhizosphere samples in the three soils, n = 24). Hereafter, we used network arithmetic, intersecting the cast and rhizosphere networks to only keep the overlapping correlations in common between these two microhabitats (Fig.S5, Phase 3). Last, we removed bulk-specific correlations from the rhizosphere-cast intersected network by subtracting correlations seen in the bulk network (Fig.S5, Phase 4). The final network so-obtained reflects OTUs whose correlations patterns are strictly specific to both casts and rhizospheres. The network was visually organized into clusters based on the z-score level of each OTUs using hierarchical clustering (Fig.S6).

3. Results

3.1 Plant traits

Earthworm presence resulted in a systematic increase in dry shoot weight in all tested soils ($p < 0.05$; Tab.2). Height and leaf surface were also increased in clay and sand soils, but not in the loam one. Shoot biomass was increased on average by +21%, while height and leaf surface area were respectively increased by 5% and 11% except in the loam soil.

3.2 Beta-diversity

Phylogenetic analysis of 16S rRNA gene amplicon profiles showed a strong soil effect (Fig.2, Tab.S1). Therefore a refined beta-diversity analysis was done for each soil separately to focus on microhabitats (Fig.3). Rhizosphere communities always differed from those of other microhabitats (Fig.3 axis 1, 18-24%) with distinct taxonomic composition at phylum level (Fig.2), while cast communities where more similar to those from bulk soils (Fig.3 axis 2, 4-8% and Fig.2). Bulk and cast communities in the earthworm treatment clustered away from the control bulk in the clay soil (yellow apart from blue, Fig.3a). However, bacterial communities in bulk and cast were similar to the control in the sand soil (yellow close to blue, Fig.3c). The loam soil had an intermediate profile (Fig.3b).

The simultaneous presence of plant and earthworm changed community profiles in cast and rhizosphere (Fig.2-3). Earthworms influence on rhizosphere communities was always detected, (Fig.3 axis 1-2, green and red squares) due to an increase of earthworm-responding rhizosphere OTUs up to ~3-folds, being mainly affiliated to Bacteroidetes, Beta- and Gammaproteobacteria (Fig.2, Fig.4a, Fig.S2-S3). Plant influence on cast communities was also always detected, although weaker in the clay soil (Fig.2-3, axis 2, yellow and red triangles), with an average abundance increase of plant-responding cast OTUs ranging from ~2-folds in the clay soil up to ~9-folds in the sand soil, being mainly affiliated to Alpha-, Betaproteobacteria, Verrucomicrobia and Bacteroidetes (Fig.4b, Fig.S2-S3). Similarly, we identified responding OTUs whose abundance was decreased by the addition of the other macroorganism in rhizospheres (Fig.4c) and casts (Fig.4d). When looking at the phylogenetic origin of these responding OTUs in all soils (Tab.S2), we noticed that 6 families were systematically responding in all soils, both in casts and rhizospheres, either with increased abundance (Bacteroidetes families: Cytophagaceae, Flavobacteriaceae, unclassified Sphingobacteriales; Betaproteobacteria families: Oxalobacteraceae, Comamonadaceae), or decreased abundance (Alphaproteobacteria: Sphingomonadaceae) when both macroorganisms were present.

3.3 Tracing back origins of bacterial OTUs

We searched for a shared core microbiota of OTUs present both in casts and rhizospheres from single and both macroorganisms treatments using a Venn diagram. Only cosmopolitan OTUs strictly found in all soils at least in 75% of biological replicates (3/4) were considered. A total of 366 OTUs were retained, featuring distinct endemic fractions found only when one or two macroorganisms were present either in the cast and rhizosphere (Fig.5a). Nevertheless, 73 core OTUs (20%) were commonly found everywhere. This number extended to 106 (white numbers) when considering only conditions with both macroorganisms. These 106 OTUs were dominated by Actinobacteria and Proteobacteria (Fig.5b, pie chart), showing a significant phylogenetic clustering (Tab.S3).

To better identify OTUs specific from the plant-earthworm interaction, a “source-sink” plot was established to trace OTU origins based on pure presence/absence patterns using cosmopolitan OTUs found in 75% of biological replicates (3/4) in all three soils. (Fig.5c). We hypothesized that sources of bacteria for rhizospheres (g) and casts (h) in the presence of both macroorganisms were following a hierarchical priority order: i) the control bulk soil without macroorganisms (a); ii) the microhabitat created by each macroorganism alone (b for g and c for h); iii) the second microhabitat created by the other macroorganism alone (c for g and b for h); iv) the other microhabitat when both macroorganisms were present (h for g and g for h); v) the bulk soil sampled next to the microhabitat of the macroorganism alone (d for g and e for h); vi) the bulk soil surrounding the microhabitat of the other macroorganism alone (e for d and b for h); vii) the bulk soil sampled when both macroorganisms were present (f); viii) the remaining parts were thus specifically attributed to each microhabitat as endemic fractions resulting from the interaction (g and h). The main source of bacteria was the bulk soil without any macroorganism (a: 65% in g and 72% in h), followed by microhabitats (bc, 20% in g; 13% in h) and other bulk soils (def, 2% in g; 6% in h). A prevalent contribution of plant rhizospheres (b: 18% in g, 6% in h) compared to earthworm casts (c: 7% in h, 2% in g) was observed. This approach evidenced endemic fractions only seen in microhabitats with

both macroorganisms (g: 14%; h: 9%), which correspond to the core microbiota of plant-earthworm interaction respectively in rhizospheres and casts.

3.4 A core microbial network

Going beyond presence/absence patterns identified with the source-sink approach, we wondered if the core microbiota could also be characterized based on OTUs abundance and co-occurrence patterns using a network analysis. We thus tailored a unique, custom approach to seek for the existence of a core microbiota network specific of the plant-earthworm interaction across soils (as explained in Fig.S5). For this purpose, we focused only on cosmopolitan OTUs sharing simultaneously strong Spearman correlations ($r > |0.6|$, FRD-adjusted q-values < 0.05) in both casts and rhizospheres, but not in the bulk soil (Fig.S5).

Indeed, we could detect such a network, made of OTUs whose abundance correlation patterns were conserved and strictly specific of all tested casts and rhizospheres (Fig.6). Similarly to the Venn diagram, the network was also showing a significant phylogenetic signal (Tab.S3) due to the same dominance of Actinobacteria and Proteobacteria. Based on hierarchical clustering of OTUs standardized abundance (Fig.S6), this network could be organized into three distinct clusters, marked by the different node shape in the network (diamond, circle and triangle of Fig.6). OTUs in each cluster displayed significant abundance patterns depending on soil type, as summarized in the upper-right bar chart (Fig.6). Respectively, the diamond-shaped OTUs were increased due to macroorganisms presence in the sand soil, but decreased in the clay soil, while not changing in the loam soil. The circle-shaped OTUs had the opposite pattern, increasing in abundance in the clay soil, but decreasing strongly in the sand soil, and slightly in the loam soil. The triangle-shaped OTUs always had significantly increased abundance in all soils under macroorganisms presence, especially in the loam soil.

3.5 Molecular abundances of archeae, fungi and bacteria

We investigated if the mutual presence of a plant and earthworms could impact the abundance of other microbial groups by qPCR. Data normality was assessed with d'Agostino test on the residuals of each ANOVA model ($p > 0.05$). Outlier values were removed based on ANOVA diagnosis plots (3/96, 4/96 and 3/96 values for bacteria, fungi and archaea respectively), leaving 3-5 biological replicates per condition. To account for the strong soil and macroorganism effects, rhizosphere and cast datasets were standardized to z-scores using their respective bulk soils under the macroorganisms presence (plant bulk for the rhizosphere, earthworm bulk for the cast, and plant-earthworm bulk for both casts and rhizosphere when both are present). Statistical significance against their bulk soils and between microhabitats was tested with Student tests (two-sample, one-sided, $p < 0.05$). The one-side version of the test was selected because we hypothesized that macroorganisms will have positive effects on microbial abundances. The presence of one or both macroorganisms resulted in significant abundance increase for all tested groups (Fig.7, stars above vertical bars), especially in the sand soil witch had the most significant hits (all rhizospheres with earthworms + bacteria and fungi in casts with plant + the fungi in casts), followed by loam soil (bacteria and fungi in all rhizospheres with or without earthworms), and clay soil (fungi in all rhizospheres with or without earthworms). Furthermore, adding the second macroorganism resulted in significant increase of microbial abundances (Fig.7, stars above horizontal lines), again with more significant differences in the sand soil (all rhizospheres with earthworms), followed by loam soil (fungi in rhizospheres with earthworms), and none in the clay soil.

4. Discussion

4.1 The core microbiota of earthworms and plants

So far, no studies reported the existence of a core microbiota for a given earthworm species across different soil types. In fact, not all macroorganism host have a core microbiota, as several examples of species were reported to live without a stable resident microbiota because of specific traits such as fast transit in short digestive tracks (e.g. caterpillars, for more examples see Hammer *et al.* 2017, 2019). As earthworms rely on fast digestion to both feed and progress in soil *via* their short tubular

guts, one could speculate that they do not have a stable resident microbiota as well. The beta-diversity analysis revealed clearer community differentiation for rhizospheres compared to casts relative to the bulk (Fig.2-3). Our observation was in line with the expectation of a slight effect of the short digestive track of earthworms, as compared with the necessity for plants (which are sessile organisms whose survival strongly depends on successful implantation) to actively recruit beneficial microbes (Tkacz & Poole 2015; Pérez-Jaramillo *et al.* 2016; Finkel *et al.* 2017). Nevertheless, we showed for the first time that a core microbiota of 136 OTUs in the casts left by the earthworm *Aporrectodea caliginosa* could be found across different soils, (cast intercept, Fig.5a). As most of these OTUs were detected in the control bulk soils (Fig.5c), this led us to assume that earthworms evolving through a soil matrix do select, in a conserved manner, the same microbial species present in different environments. Regarding plant core microbiota, we evidenced here the presence of a rhizospheric core microbiota of 113 OTUs for *Hordeum vulgare*, found across all tested soils (rhizosphere intercepts, Fig.5a). This is consistent with other studies identifying a rhizospheric core microbiota occurring independently of environmental context (Lundberg *et al.* 2012; Chowdhury *et al*. 2019). As most of these OTUs were also detected in the control bulk soils (Fig.5c), this also suggests that plants selected a set of ubiquitous bacterial species present in different environments. Additional experiment would be required to reinforce and support the notion of a core microbiota for earthworms by adding more soils, and also investigate the proportion of OTUs that could be originating from host themselves (earthworm's gut and plant seed endophytes).

4.2 A core microbiota resulting from plant-earthworm interaction

By definition, core microbiota are identified across a range of various environmental conditions defined by abiotic parameters like soil type for plants (Lundberg *et al.* 2012; Chowdhury *et al*. 2019) or diet for mammals (Turnbaugh *et al.* 2009). However, they may also be influenced by biotic factors, for instance the presence of another organisms (e.g. intestinal parasites, Leung *et al.* 2018). This becomes crucial for external microbiota, as they are directly exposed to other

macroorganisms (e.g. earthworms influencing rhizosphere microbiota, Braga *et al.* 2016). Moreover, if the other macroorganism is also harboring an external microbiota, an interaction may occur. This becomes critical when both hosts share the same habitat, like earthworms and plants overlapping there rhizosphere and drilosphere in soils (Lavelle 2002; Kuzyakov & Blagodatskaya 2015), or rhizosphere and cuticle microbiota of nematodes that may shuttle pathogens (Elhady *et al.* 2017).

In this study, we successfully evidenced a reciprocal influence of earthworms and plants on their respective functional domains through the prism of microbiota, which was characterized by two major findings. The first one was the systematic presence of responding OTUs in casts and rhizospheres whose abundance was altered by the presence of the other macroorganism (Fig.4, Tab.S2). These OTUs were related to known poaceae rhizospheric taxa (Bulgarelli *et al.* 2015) such as members from Comamonadaceae (Betaproteobacteria) and Flavobacteriaceae (Bacteroidetes), but also others like Sphingobacteriaceae (Alphaproteobacteria), Oxalobacteraceae (Betaproteobacteria), Cytophagaceae and Flavobacteriaceae (Bacteroidetes), which are often referenced as being of importance for plant health (Yin *et al.* 2013; Hassani *et al.* 2018; Schlatter *et al.* 2019). These responding OTUs might be potential plant beneficial microbes stimulated by earthworms, hence leading to the increased plant growth observed with earthworms (Tab.2). However, their reciprocal presence and importance in the casts of earthworms remain unresolved.

The second one was the detection of 106 unique endemic OTUs that became detectable in both casts and rhizospheres only when the two macroorganisms were present (Fig.5a), with a strong phylogenetic signal in favor of Actinobacteria and Proteobacteria (Fig.5b, Tab.S3), which can be considered as a strong evidence for the presence of a core microbiota resulting from the interaction between plants and earthworms. A first, the concept of microbial community coalescence (Rillig *et al.* 2015), introduced to define the forming of a novel microbial community (C) from the joining of two previously separated ones (A and B), seemed relevant to understand our results. However this notion does not fully match our situation, where there is a pre-existing soil environmental matrix

from which ecosystem engineers sample the same microbial pool. Our data suggest no convergence between rhizosphere and cast community structures (Fig.3), as it could be expected from the coalescence theory. Moreover, the Venn diagram clearly indicated that previously unseen and unique OTUs were systematically detected in each microhabitat in the presence of the second macroorganism (cast: 42; rhizosphere: 27, Fig.5a), indicating a situation where A and B respectively become A' and B' rather than a composite C. Consequently, our results are discussed with a different meta-community framework accounting for the existence of an environmental matrix: the source-sink model (Mouquet & Loreau 2003; Lindegren *et al.* 2014). We applied it for tracing respective contributions of several sources to the constitution of sinks, i.e. rhizosphere and cast, when both macroorganism species are present (Fig.5c). This approach showed the major contribution of the bulk soil for casts (72%) and rhizospheres communities (65%). OTUs were also found in the rhizosphere only when earthworms were present (14%) or in the casts only when plants were present (9%), which could be due for example to the release of organic compounds by the second macroorganism (e.g. root exudates by plants or mucus by earthworms). The processes behind the crossed-contribution of these two major soil ecosystem engineers to the selection of a specific core microbiota reflecting their interaction in all tested soils deserve more attention, as it may have important functional and evolutionary implications that have yet to be found. The fact that our plant biomass was increased under the presence of earthworms (a well-established observation in meta-analyses; van Groenigen *et al.* 2014; Blouin *et al.* 2019), seems to indicate that functional and adaptive mechanisms are at play. As plants and earthworms shared the same soils and microbial dwellers over several hundred million years (Anderson *et al.* 2017; Harrison *et al.* 2018), it can be speculated that a certain degree of co-evolution occurred between these two ecosystem engineers (Blouin, 2018), in which soil microbes may play a key role. Although the phylogenetic signal identified in this study brings a first element in this direction, additional work would be needed to investigate it. As a perspective, it would be interesting to confirm that the plant influence is systematic on the casts, while the influence of earthworms on the rhizosphere is soil-

dependent, as observed in our results. The weak effect of the plant on cast microbiota in the clay soil, which was stronger in the loam and sand soils (Fig.3-4) could be interpreted as the result of adaptive mechanisms related to soil fertility/texture.

4.3 A network emerging from the core microbiota of plant-earthworm interactions

Going beyond the mere presence/absence aspects required to identify a core microbiota, we introduced in this study a novel concept that we referred to as a core microbiota network, bringing an additional level of information to characterize a core microbiota (Fig.6). To our knowledge, this procedure has never been used to describe a core microbiota before. Using a custom approach, we were able to detect the presence of such a core microbiota network, made of OTUs whose correlation patterns are only seen in both casts and rhizosphere, but not in the bulk of all tested soils. Similarly, a significant phylogenetic signal in favor of Proteobacteria and Actinobacteria has been identified (Tab.S3), thus supporting the hypothesis that a specific microbial assemblage resulting from the plant-earthworm interaction occur in those microhabitats. This network was organized into three clusters of OTUs depending on how they reacted to the mutual presence of both macroorganisms in the three tested soils. It is worth noticing that two clusters displayed opposite patterns that seemed to depend on the soil properties (texture and/or fertility), being i) the diamond-shaped OTUs with gradual enhanced abundance from clay, loam up to sand soils, and ii) circle-shaped OTUs with the converse enhanced trend from sand, loam up to clay soil (Fig.6, upper-right bar chart). Additional analysis would be required to confirm if soil properties are indeed shaping this core microbiota network. The last cluster features OTUs whose abundance are always increased in all soils (especially in the loam soil), thus representing a good basis for the definition of potential microbial indicator of the plant-earthworm interaction. Altogether, these microbial observations are questioning on the existence of something emerging from the plant-earthworm interaction in soils (e.g. creation of a novel microbial niche; Odling-Smee *et al.* 1996; Matthews *et al.* 2014).

4.4 Effect of plant-earthworms interaction on abundances of bacteria, fungi and archaea

Our qPCR results show that the outcome of this interaction is not restricted to bacteria. Indeed, earthworms stimulated fungi in loam soil rhizospheres as well as all microbial domains in sand soil rhizospheres, while bacteria were promoted in sand soil casts by plants (Fig.7). These results suggest that effects of multiple host interaction may impact the whole soil microbial community. Thus, microbial "hotspots" and "hot moments" (Kuzyakov & Blagodatskaya 2015) are dependent on macroorganisms interaction and on soil properties (Fig.7). Hotspots and hot-moments could be more frequent when benefiting from several energy sources (earthworm mucus and plant exudates), especially in sand soil, the poorest in organic matter, in which ecosystem engineer activities could be a major determinant of soil function. Again, results indicate that effects of macroorganisms on microbial abundances is soil dependent, questioning on the fact that soil properties might modulate the plant-earthworm interaction at the microbial level.

5. Conclusion

Altogether, results indicated a joint shaping of microbial communities by plants and earthworms, correlating with an increase in plant biomass. This interaction resulted in the emergence of i) core microbiota specific of plant-earthworm interaction across soils, revealed in both casts and rhizospheres, as well as ii) a core microbiota network being specific of the casts and rhizosphere whose OTUs clustering was indicative of soil type. These results are opening the path for future research on the role of this core microbiota in plant-earthworm interactions. An immediate perspective would be to better characterize this core microbiota of plant-earthworm interaction through a functional approach, in accordance with the concept of functional core microbiota (Lemanceau *et al.* 2017).

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Authorship statement

Authors declared no conflicting nor competing interests, and approved the content of this work. Contributions: SJ (analytical strategy, data analysis, manuscript writing), RPF (laboratory experiment, manuscript editing), CMon (sequencing strategy), CMou (sequencing strategy), AS (analytical strategy, data analysis, manuscript editing), AM (bioinformatic), LP (analytical strategy, manuscript editing), MB (study conception, research direction, analytical strategy, manuscript writing). Correspondence and requests for materials should be addressed to manuel.blouin@agrosupdijon.fr.

Data availability statement

Data that support the findings of this study have been deposited in the Sequence Read Archive database (SRA, https://www.ncbi.nlm.nih.gov/sra) with the primary accession code "SUB5123378", and will be made automatically publically available either after publication or after the embargo deadline. A temporary link may be provided upon request. Upon acceptance, the final accession number will be added at the end of the article. The Rgui software and associated function packages used for data analysis are all publically available (R Development Core Team. R. A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna: RC Team; 2017. http://www.R-project.org).

Figure legends

Fig.1: Experimental design. Three soils (sand, loam, clay) were used in four treatments (no macroorganisms, earthworms, plant, both) and replicated five times, giving 60 microcosms. Plant traits were recorded on each microcosms (n = 60). Soil microhabitats were sampled depending on the treatment, yielding 40 samples per soil type (20 bulks, 10 casts and 10 rhizospheres), representing a total of 120 samples (3 soils x 40 microhabitats). qPCR assay was done on all samples (n = 120) while 16S rRNA gene amplicon sequencing was done only on four biological replicates, excluding the outlier-most sample based on plant traits (n = 96).

Fig.2: Averaged phylogenetic overview of bacterial communities detected in bulk, cast and rhizosphere samples in all soils and treatments. Horizontal bar chart was obtained by averaging the relative abundances across the biological replicates for each microhabitat at phylum level (down to class level for Proteobacteria). Cluster dendrogram was established with the *Manhattan* distance and *complete* method.

Fig.3: Distance-based redundancy analysis showing the principal constrained coordinates of bacterial communities in each soil (weighted unifrac distances, 10.000 permutations). The four treatments are indicated by different colors, while microhabitats are indicated by different marker shape.

Fig.4: Responding OTUs reacting to the second macroorganisms in casts and rhizospheres (Fig.S2-Fig.S3 for details). Panel a and b are showing OTUs whose abundance was increased in rhizospheres and casts when earthworms and plants were added respectively (w = with; w/o = without; ew = earthworms; pl = plant). Panel c and d are showing OTUs whose abundance was decreased in rhizospheres and casts when earthworms and plants were added respectively

Fig.5: Core microbiota of the pant-earthworms interaction. Only OTUs found at least in 75% of the biological replicates (3/4) and in all soils were considered (n = 465). Panel a shows a Venn diagram depicting the core microbiota shared between casts and rhizosphere when both macroorganisms are present (in white, n = 106). Panel b shows the unweighted taxonomic distribution of the 106 OTUs, mainly dominated by Actinobacteria and Proteobacteria. Panel c shows the origin of OTUs in a source-sink plot from “sources” (a-f) going into “sinks” (rhizosphere “g” and cast “h” with both macroorganisms). A hierarchical sorting rule was applied to attribute OTU sources for each sink (g: {abchdef}; h: {acbgedf}). Percentages on arrows indicate source contributions.

Fig.6: Core microbiota network of OTUs found in cast and rhizosphere of all soils, but not in the bulk. Only OTUs found in 50% of replicates in each soil were included. The strategy

656 describing the network analysis is presented in supporting data (Fig.S5). Cluster membership (node
657 shapes) is based on OTU standardized abundances against control bulk (upright bar chart, average
658 z-score ± standard error of the mean, n = 48). With both macroorganisms, diamond-shaped OTUs
659 had increased abundance in sand soil but decreased in clay soil. A converse pattern was observed
660 for circle-shaped OTUs. Triangle-shaped OTUs increased everywhere, especially in the loam soil.

661 **Fig.7:** qPCR estimation of bacterial/archaeal genetic markers (a and b, 16S rRNA gene) and
662 fungi (c, ITS). Molecular copy counts were standardized against average and standard deviation
663 values of reference bulk soils from the same treatment (z-score). Bar charts are representing z-score
664 averages ± standard error of the mean (n = 3-5). Significance between treatments were assessed by
665 two-sample, one-sided Student tests (top horizontal lines between treatments). Significance relative
666 to the reference bulk (zero-baseline) were assessed by one-sample, one-sided Student tests
667 (indications above bars). Significance: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; . $p > 0.1$.

668

669 **Tables**

670 **Tab.1:** Characteristics of the three contrasting soils used in this study to set microcosms.

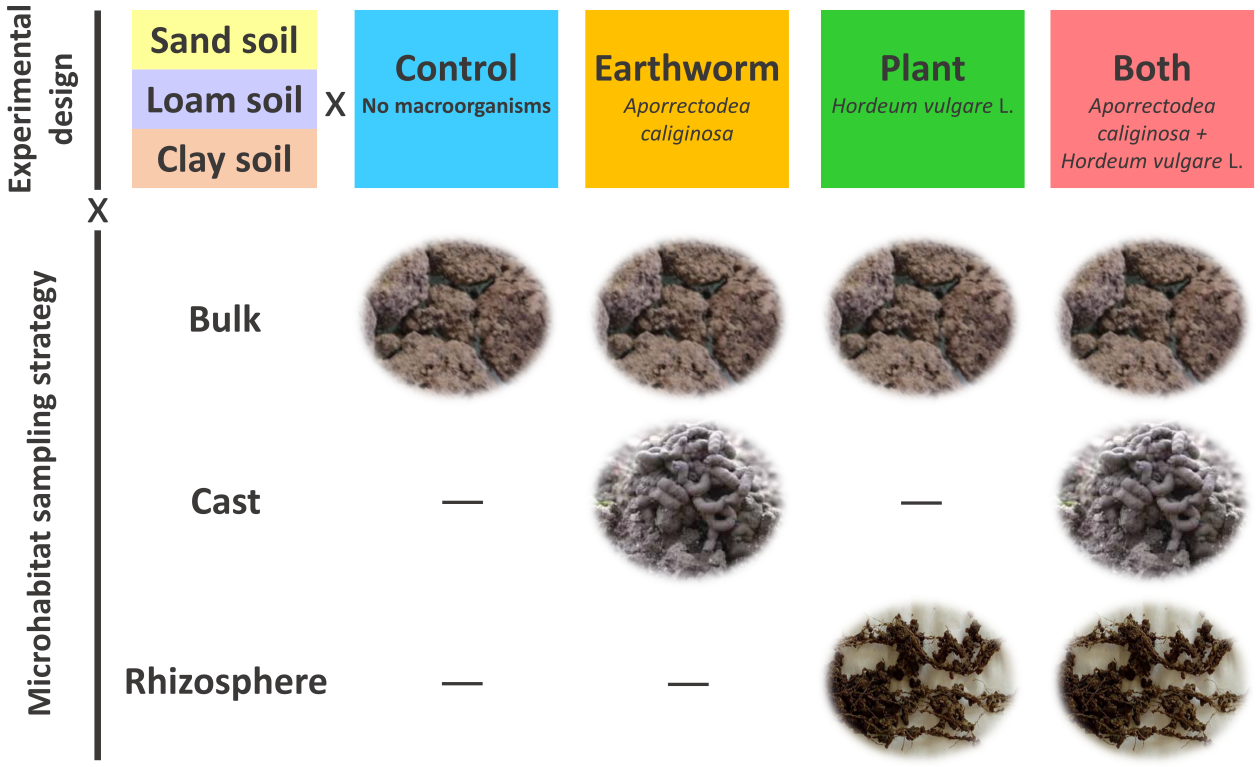
Description	C org.	N tot.	pH	Clay	Loam	Sand	Name
Cambisoil with moor	14.7	1.2	5.2	6.9%	19.0%	74.1%	Sand soil
Cropping luvisoil	9.2	0.9	7.0	16.7%	56.2%	27.1%	Loam soil
Forest leptosoil	56.7	4.7	7.5	34.4%	39.2%	27.4%	Clay soil

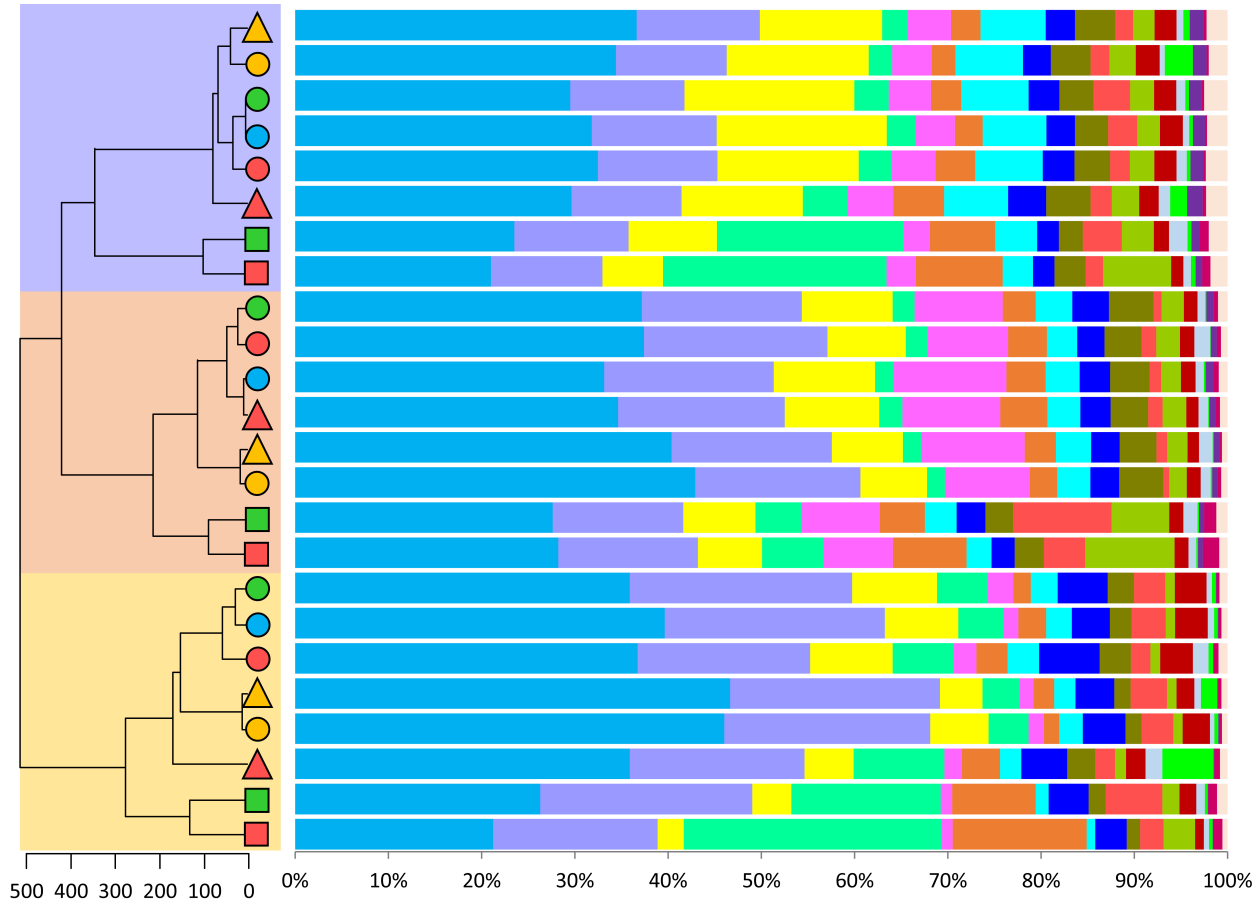
671

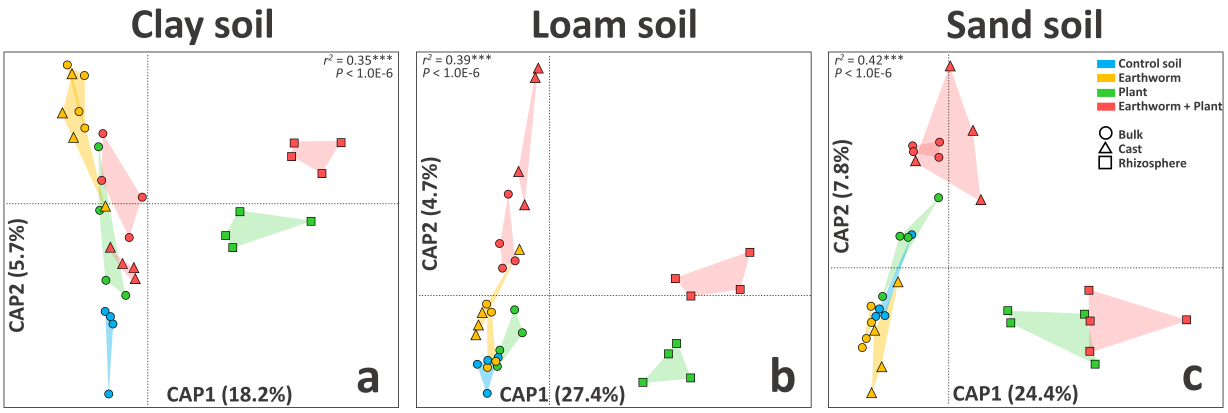
672 **Tab.2:** Effect of earthworms on barley traits at harvest in each soil. Data were collected only
673 for the treatments that had plants, with or without earthworms (green and red treatments, Fig.1).
674 Three traits were measured (rows), including height (the longest leaf length, which was always the
675 highest in our case), dry shoot weight and leaf surface area. Statistical significance was tested using
676 two-sided, two-sample Student tests ($p < 0.05$) to compare average values (± standard error of the
677 mean) between the condition without (-) and with (+) earthworms. Lowercase letters indicate
678 statistically significant difference between tested average values ("a": highest, "b": lowest). All
679 tested conditions were set with five biological replicates.

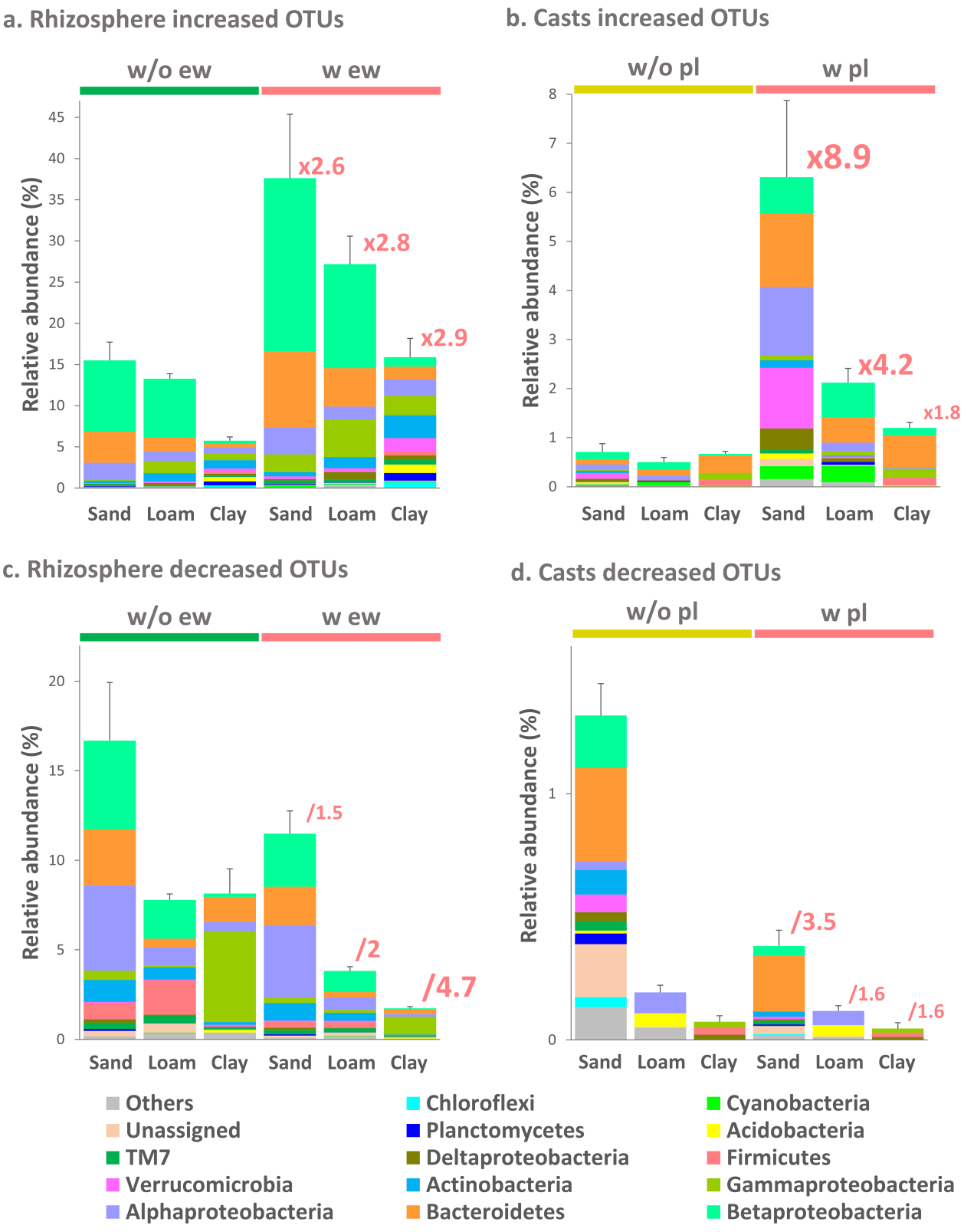
Soil	**Clay**		**Loam**		**Sand**	
Earthwoms	**-**	**+**	**-**	**+**	**-**	**+**
Height (mm)	34.80 ±0.26^{b}	37.10 ±0.8^{a}	30.30 ±0.51	31.10 ±0.6	32.10 ±0.56^{b}	34.3 ±0.44^{a}
Dry weight (g)	0.44 ±0.01^{b}	0.60 ±0.05^{a}	0.32 ±0.03^{b}	0.370 ±0.03^{a}	0.36 ±0.01^{b}	0.44 ±0.02^{a}
Surface (mm^2)	47.59 ±2.88^{b}	54.20 ±2.51^{a}	36.22 ±2.32	35.50 ±2.26	41.63 ±1.83^{b}	47.33 ±2.49^{a}

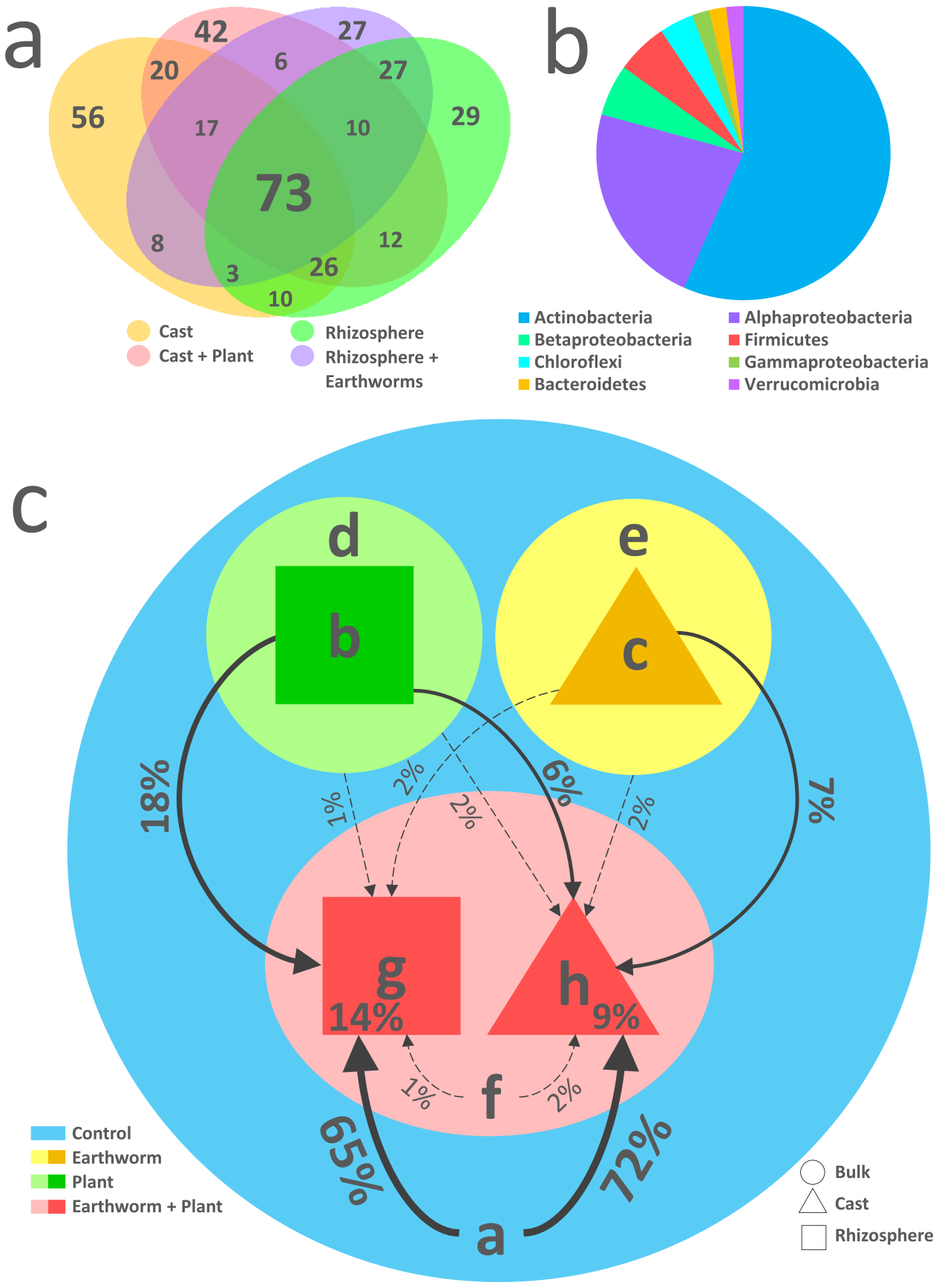
680

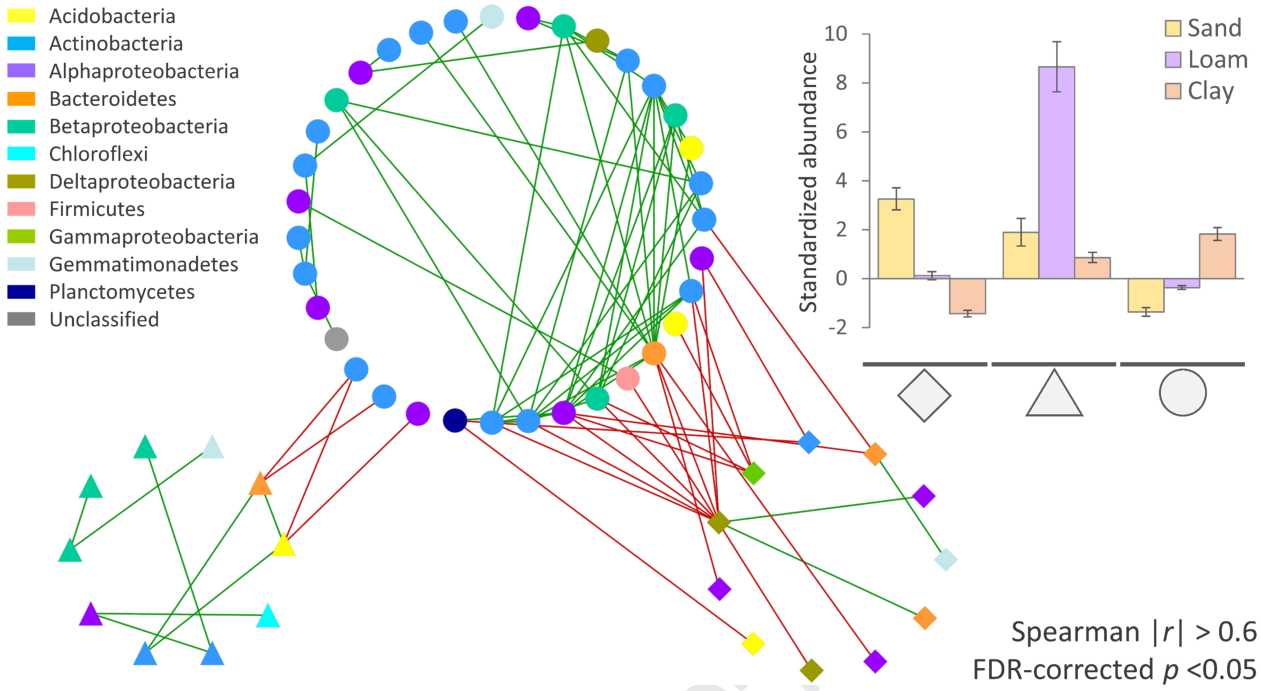


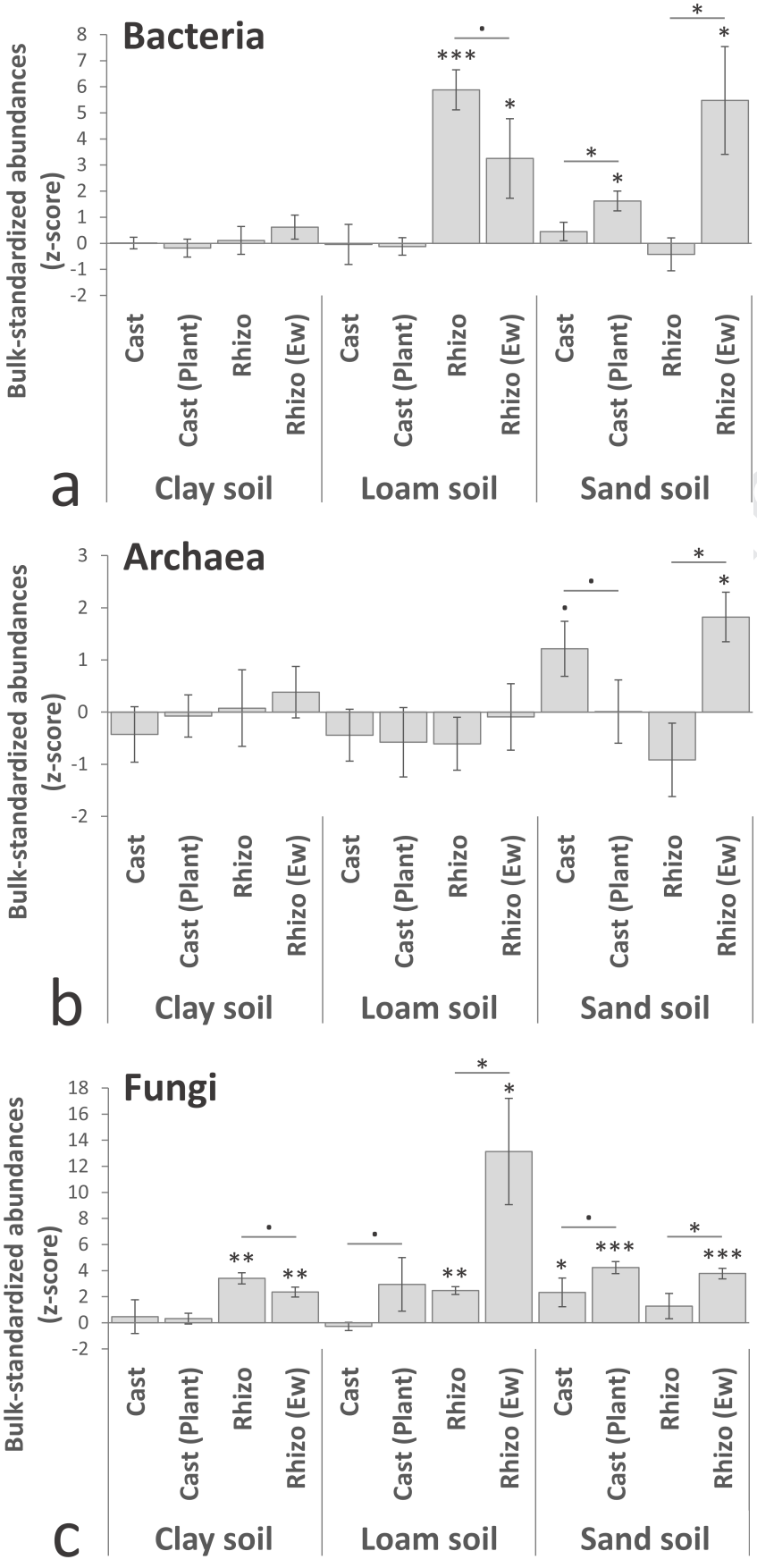

500 400 300 200 100 0
Manhattan distance
0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%
Relative abundance (%)
Sand soil
Loam soil
Clay soil
Control soil
Earthworm
Plant
Earthworm + Plant
Bulk
Cast
Rhizosphere
Actinobacteria
Acidobacteria
Verrucomicrobia
Chloroflexi
Deltaproteobacteria
Gammaproteobacteria
Unassigned
Nitrospirae
Others
Alphaproteobacteria
Betaproteobacteria
Bacteroidetes
Planctomycetes
Firmicutes
Gemmatimonadetes
Cyanobacteria
TM7




a. Rhizosphere increased OTUs
w/o ew
w ew
Relative abundance (%)
x2.6
x2.8
x2.9
Sand
Loam
Clay
b. Casts increased OTUs
w/o pl
w pl
x8.9
x4.2
x1.8
c. Rhizosphere decreased OTUs
/1.5
/2
/4.7
d. Casts decreased OTUs
/3.5
/1.6
/1.6
Others
Unassigned
TM7
Verrucomicrobia
Alphaproteobacteria
Chloroflexi
Planctomycetes
Deltaproteobacteria
Actinobacteria
Bacteroidetes
Cyanobacteria
Acidobacteria
Firmicutes
Gammaproteobacteria
Betaproteobacteria


a
42
27
20
6
27
56
17
10
29
73
8
12
3
26
10
Cast
Cast + Plant
Rhizosphere
Rhizosphere + Earthworms
b
Actinobacteria
Alphaproteobacteria
Betaproteobacteria
Firmicutes
Chloroflexi
Gammaproteobacteria
Bacteroidetes
Verrucomicrobia
c
d
b
e
c
18%
1%
2%
2%
6%
2%
7%
g
14%
h
9%
f
1%
2%
65%
72%
a
Control
Earthworm
Plant
Earthworm + Plant
Bulk
Cast
Rhizosphere




Bacteria
Bulk-standardized abundances (z-score)
8
7
6
5
4
3
2
1
0
-1
-2
***
*
*
*
*
*
Cast
Cast (Plant)
Rhizo
Rhizo (Ew)
Cast
Cast (Plant)
Rhizo
Rhizo (Ew)
Cast
Cast (Plant)
Rhizo
Rhizo (Ew)
a
Clay soil
Loam soil
Sand soil
Archaea
Bulk-standardized abundances (z-score)
3
2
1
0
-1
-2
*
*
Cast
Cast (Plant)
Rhizo
Rhizo (Ew)
Cast
Cast (Plant)
Rhizo
Rhizo (Ew)
Cast
Cast (Plant)
Rhizo
Rhizo (Ew)
b
Clay soil
Loam soil
Sand soil
Fungi
Bulk-standardized abundances (z-score)
18
16
14
12
10
8
6
4
2
0
-2
**
**
**
*
*
***
***
Cast
Cast (Plant)
Rhizo
Rhizo (Ew)
Cast
Cast (Plant)
Rhizo
Rhizo (Ew)
Cast
Cast (Plant)
Rhizo
Rhizo (Ew)
c
Clay soil
Loam soil
Sand soil

A core microbiota of the plant-earthworm interaction conserved across soils

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Highlights:

1. A core microbiota of the plant-earthworm interaction was identified in all soils
2. The core microbiota is present in earthworm casts and plant rhizospheres
3. A core microbiota network specific of casts and rhizosphere was detected
4. Bacteria, fungi and archeae are affected by the plant-earthworm interaction
5. Soil type modulates plant-earthworm interaction on microbial communities
6. This core microbiota fosters revisiting aboveground-belowground interactions

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: