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

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Abstract

10
11 The core microbiota defines the fraction of microorganisms common to all individuals from the
12 same host species regardless of the abiotic context, be they located inside (e.g. animal guts) or
13 outside (e.g. plant rhizospheres). While the core microbiota of many host species have been
14 documented, no studies attempted to decipher how these core microbiota could be altered when
15 their respective host species are interacting. We thus tested the hypothesis that interactions between
16 different host species possessing external microbiota could result in a novel emerging entity: a core
17 microbiota of an interaction. This is particularly true in soils, where such interactions are likely to
18 occur between different host species harboring external microbiota, like plants through rhizospheres
19 and earthworms through drilospheres. Using three contrasting soils (sand, loam or clay) and a
20 meticulous sampling of different microhabitats (rhizospheres, casts and bulk) coupled to a “source-
21 sink approach” derived from the meta-community theory, we evidenced the presence of a conserved
22 core microbiota of bacterial OTUs resulting from plant-earthworm interactions in all soils. This
23 interaction was also evidenced using a tailored network analysis, revealing the presence of signature
24 OTUs always found in earthworms casts and plant rhizospheres, and whose co-occurrence patterns
25 were indicative of soil type. Furthermore, qPCR abundance estimates revealed that not only
26 bacteria, but also fungi and archaea, are affected by plant-earthworm interactions. Our findings
27 provide a new framework to explore aboveground-belowground interactions through the prism of
28 microbial communities.

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30

31 1. Introduction

32 Microorganisms participate in crucial soil functions and services like biogeochemical cycles,
33 bioremediation and food production. Their activities strongly depend on soil ecosystem engineers
34 (Jones *et al.* 1994; Lavelle *et al.* 1997) which “directly or indirectly modulate the availability of
35 resources to other species, by causing physical state changes in biotic or abiotic materials”. As a
36 consequence, soil is made of “microbial hotspots” (Kuzyakov & Blagodatskaya 2015), previously
37 described as specific “functional domains”, defined as “parts of the soil that are influenced by a
38 major biotic or abiotic regulator, [...] recognizable in a set of structures (pores, aggregates, fabrics
39 etc.) generated by the regulator that can be physically separated from the soil matrix” (Lavelle,
40 2002). In the case of plants and earthworms, two major soil ecosystem engineers, these functional
41 domains are respectively named the rhizosphere and drilosphere, which are also microhabitats for
42 the myriads of microorganisms populating them, being the microbiota. In this study, not only we
43 investigated how plants and earthworms do influence soil microbial communities in their respective
44 functional domains, but also how their interaction can reciprocally alter the microbiota residing in
45 these two microhabitats.

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

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

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

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

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

94 2. Materials and methods

95 2.1 Experimental design and sampling

96 Three soils were used (Tab.1): a sand soil (cambisol with moor from CEREEP station,
97 Saint-Pierre-Lès-Nemours, France), a loam crop soil (luvisol from INRA, Versailles, France) and a
98 forest clay soil (leptosol from MNHN station, Brunoy, France). The first 20cm were sampled
99 excluding plant material. Soils were air-dried, sieved (2mm) and set in microcosm pots of 11
100 containing 1kg of soil watered at 80% of their respective water holding capacity, being the optimum
101 for plant and earthworm (Lavelle 1978). Four conditions were tested with five biological replicates
102 (Fig.1): i) a plant, ii) three earthworms, iii) both together and iv) nothing (control). Barley
103 (*Hordeum vulgare* L. commercial variety, “La fermette”) was germinated in three batches of 80
104 seeds in Petri dishes containing humidified soil (100%, 20°C, phytotron, seven days). ~8cm-tall
105 seedlings were transplanted in pots. The earthworm *Aporrectodea caliginosa* was chosen for its
106 endogeic lifestyle. Individuals originated from a non-stop breeding program initiated in 2007 (IRD
107 park, Bondy, France). Three batches of young individuals were purged in their respective
108 experimental soil to prevent breeding substrate contaminations (three days). Three individuals were

109 introduced at the pot surfaces (total weight ~1g). Microcosms were incubated in a climatic chamber
110 (S10H, Conviron, Canada; 75% air humidity, 18/20°C night/day, 12h photoperiod with constant
111 300 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, 28 days). Leaf surface was estimated after 17 days by summing leaf
112 areas (leaf area = leaf length x mid-section leaf width x 0.75, Blouin *et al.* 2007). Plant height was
113 estimated after 23 days based on the longest leaf. After 28 days, all microcosms were used for
114 destructive sampling. Soil was meticulously sampled to recover the distinct microhabitats:
115 rhizospheric soil (adhering root soil recovered from vigorous shaking with distilled water, then
116 centrifuged), earthworm casts (visual identification; Velasquez *et al.* 2007) and bulk soil (no visible
117 macroorganisms influence). Shoot biomass was measured after drying (50°C, 48h).

118 2.2 qPCR settings

119 Total DNA was extracted from 250 mg of soil using FastDNA® SPIN Kit for Soil (MP
120 Biomedicals). DNA concentration was quantified using Quant-iT™ dsDNA High-Sensitivity Assay
121 Kit (Invitrogen) before dilution at 1 ng. μl^{-1} . Fungal ITS (ITS3F: 5'-
122 GCATCGATGAAGAACGCAGC-3', ITS4R: 5'-TCCTCCGCTTATTGATATGC-3'; White
123 1990), Crenarchaeota 16S rRNA (Crenar771F: 5'-ACGGTGAGGGATGAAAGCT-3',
124 Crenar975R: 5'- CGGCGTTGACTCCAATTG-3'; Ochsenreiter *et al.* 2003) and bacterial 16S
125 rRNA genes (341F: 5'-CCTACGGGAGGCAGCAG-3', 534R: 5'-CCTACGGGAGGCAGCAG-3';
126 Muyzer *et al.* 1993) were quantified using real-time polymerase chain reactions (qPCR) on a
127 StepOnePlus™ Real-Time PCR system (Applied Biosystem, France). Reaction mixtures (15 μl)
128 were composed of 7.5 μl Power SYBR™ Green PCR Master Mix with ROX (Applied Biosystem),
129 1.5 μl of each primer (10 μM), 2.5 μl UltraPure™ DNase/RNase-Free Distilled Water (Applied
130 Biosystem) and 2 μl diluted-DNA template. Potential inhibition was assessed by adding 2 μl of
131 known concentration of plasmid to reaction mixtures (adjusted water volume: 0.5 μl). No inhibition
132 was detected, as amplifications using primers targeting T7 and SP6 RNA polymerase promoters
133 were similar amongst samples. Amplification was set with 900 s at 95 °C for enzyme activation, 35
134 cycles of 15 s at 95 °C, 30 s at annealing temperatures (ITS/Archaea: 55°C, Bacteria: 60°C), 30 s at

135 72 °C for elongation and 30 s at 80 °C for termination. Abundances were quantified with linearized
136 plasmid-based standard curves (StepOne™ Software v2.2.2, three technical repetitions). Copy
137 numbers were normalized per µgram of DNA per gram of soil, and log₂-transformed for analysis.

138 2.3 16S rRNA gene amplicon sequencing

139 Bacteria were selected for their importance in plant-earthworm interaction (Hoeffner *et al.*
140 2018). Amplicons were generated from purified DNA by LGC Genomics (GmbH, Germany),
141 respecting best practices guidelines (Berry *et al.* 2011; Schöler *et al.* 2017). The bacterial 16S rRNA
142 gene V3-V4 hypervariable region was amplified with fusion primers U341F (5'-
143 CCTACGGGNGGCWGCAG -3') and 785R (5'- GACTACHVGGGTATCTAAKCC -3';
144 Klindworth *et al.* 2013) equipped with barcode sequences for each sample, with the following
145 protocol: 20µL reaction mixtures, 1.5 units MyTaq DNA polymerase (Bioline, Germany), 2 µl of
146 BioStabII PCR Enhancer (Sigma, Germany), 15pmol of each primer, 5 ng template DNA, 96°C for
147 2 min followed by 30 cycles (96°C for 15s, 50°C for 30sec), 70°C for 90s and termination (72°C,
148 10 min). Amplicon were visualized (2% agarose electrophoresis, ~500 bp). Two pools of 48
149 samples (20 ng each) were generated (n = 96), purified once (AMPure XP beads, Agencourt,
150 Germany) and twice (MinElute columns, Qiagen, Germany). 100 ng of purified pools were used for
151 Illumina library construction (Ovation Rapid DR Multiplex System 1-96, NuGEN, Germany).
152 Libraries were pooled and size-selected by electrophoresis. Sequencing was performed on MiSeq
153 (Illumina MiSeq reagent kit v2, 2x250 bp), followed by demultiplexing and trimming of
154 adaptors/barcodes (Illumina MiSeq Reporter software v2.5.1.3). Sequences were analyzed in-house
155 with a Python notebook. Sequences were assembled (PEAR, default settings, Zhang *et al.* 2014),
156 removing short sequences and applying quality checks (QIIME, Caporaso *et al.* 2010). Reference-
157 based and *de novo* chimera detection, and Operational Taxonomic Unit (OTU, 97%) clustering
158 were performed using VSEARCH (Rognes *et al.* 2016), and taxonomy was assigned (UCLUST,
159 Edgar 2010) with the Greengenes database (v05, MacDonald *et al.* 2012). OTU representative
160 sequences were aligned (PyNAST, Caporaso *et al.* 2011) to build a phylogenetic tree (FastTree,

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

164 2.4 Beta-diversity and multivariate analysis

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

184 2.5 Network analysis

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

204 3. Results

205 3.1 Plant traits

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

3.2 Beta-diversity

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

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

3.3 Tracing back origins of bacterial OTUs

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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

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

264 *3.4 A core microbial network*

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

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

285 *3.5 Molecular abundances of archaea, fungi and bacteria*

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

305 4. Discussion

306 4.1 *The core microbiota of earthworms and plants*

307 So far, no studies reported the existence of a core microbiota for a given earthworm species across
308 different soil types. In fact, not all macroorganism host have a core microbiota, as several examples
309 of species were reported to live without a stable resident microbiota because of specific traits such
310 as fast transit in short digestive tracks (e.g. caterpillars, for more examples see Hammer *et al.* 2017,
311 2019). As earthworms rely on fast digestion to both feed and progress in soil *via* their short tubular

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

332 4.2 A core microbiota resulting from plant-earthworm interaction

333 By definition, core microbiota are identified across a range of various environmental
334 conditions defined by abiotic parameters like soil type for plants (Lundberg *et al.* 2012; Chowdhury
335 *et al.* 2019) or diet for mammals (Turnbaugh *et al.* 2009). However, they may also be influenced by
336 biotic factors, for instance the presence of another organisms (e.g. intestinal parasites, Leung *et al.*
337 2018). This becomes crucial for external microbiota, as they are directly exposed to other

338 macroorganisms (e.g. earthworms influencing rhizosphere microbiota, Braga *et al.* 2016).
339 Moreover, if the other macroorganism is also harboring an external microbiota, an interaction may
340 occur. This becomes critical when both hosts share the same habitat, like earthworms and plants
341 overlapping their rhizosphere and drilosphere in soils (Lavelle 2002; Kuzyakov & Blagodatskaya
342 2015), or rhizosphere and cuticle microbiota of nematodes that may shuttle pathogens (Elhady *et al.*
343 2017).

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

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

364 from which ecosystem engineers sample the same microbial pool. Our data suggest no convergence
365 between rhizosphere and cast community structures (Fig.3), as it could be expected from the
366 coalescence theory. Moreover, the Venn diagram clearly indicated that previously unseen and
367 unique OTUs were systematically detected in each microhabitat in the presence of the second
368 macroorganism (cast: 42; rhizosphere: 27, Fig.5a), indicating a situation where A and B
369 respectively become A' and B' rather than a composite C. Consequently, our results are discussed
370 with a different meta-community framework accounting for the existence of an environmental
371 matrix: the source-sink model (Mouquet & Loreau 2003; Lindegren *et al.* 2014). We applied it for
372 tracing respective contributions of several sources to the constitution of sinks, i.e. rhizosphere and
373 cast, when both macroorganism species are present (Fig.5c). This approach showed the major
374 contribution of the bulk soil for casts (72%) and rhizospheres communities (65%). OTUs were also
375 found in the rhizosphere only when earthworms were present (14%) or in the casts only when plants
376 were present (9%), which could be due for example to the release of organic compounds by the
377 second macroorganism (e.g. root exudates by plants or mucus by earthworms). The processes
378 behind the crossed-contribution of these two major soil ecosystem engineers to the selection of a
379 specific core microbiota reflecting their interaction in all tested soils deserve more attention, as it
380 may have important functional and evolutionary implications that have yet to be found. The fact
381 that our plant biomass was increased under the presence of earthworms (a well-established
382 observation in meta-analyses; van Groenigen *et al.* 2014; Blouin *et al.* 2019), seems to indicate that
383 functional and adaptive mechanisms are at play. As plants and earthworms shared the same soils
384 and microbial dwellers over several hundred million years (Anderson *et al.* 2017; Harrison *et al.*
385 2018), it can be speculated that a certain degree of co-evolution occurred between these two
386 ecosystem engineers (Blouin, 2018), in which soil microbes may play a key role. Although the
387 phylogenetic signal identified in this study brings a first element in this direction, additional work
388 would be needed to investigate it. As a perspective, it would be interesting to confirm that the plant
389 influence is systematic on the casts, while the influence of earthworms on the rhizosphere is soil-

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

393 *4.3 A network emerging from the core microbiota of plant-earthworm interactions*

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

415 *4.4 Effect of plant-earthworms interaction on abundances of bacteria, fungi and archaea*

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

427 5. Conclusion

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

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446

447 7. References

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603

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606 **Authorship statement**

607 Authors declared no conflicting nor competing interests, and approved the content of this work.
608 Contributions: SJ (analytical strategy, data analysis, manuscript writing), RPF (laboratory
609 experiment, manuscript editing), CMon (sequencing strategy), CMou (sequencing strategy), AS
610 (analytical strategy, data analysis, manuscript editing), AM (bioinformatic), LP (analytical strategy,
611 manuscript editing), MB (study conception, research direction, analytical strategy, manuscript
612 writing). Correspondence and requests for materials should be addressed to
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614 **Data availability statement**

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

623 **Figure legends**

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

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

636 **Fig.3:** Distance-based redundancy analysis showing the principal constrained coordinates of
637 bacterial communities in each soil (weighted unifracs distances, 10,000 permutations). The four
638 treatments are indicated by different colors, while microhabitats are indicated by different marker
639 shape.

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

645 **Fig.5:** Core microbiota of the pant-earthworms interaction. Only OTUs found at least in
646 75% of the biological replicates (3/4) and in all soils were considered (n = 465). Panel a shows a
647 Venn diagram depicting the core microbiota shared between casts and rhizosphere when both
648 macroorganisms are present (in white, n = 106). Panel b shows the unweighted taxonomic
649 distribution of the 106 OTUs, mainly dominated by Actinobacteria and Proteobacteria. Panel c
650 shows the origin of OTUs in a source-sink plot from “sources” (a-f) going into “sinks” (rhizosphere
651 “g” and cast “h” with both macroorganisms). A hierarchical sorting rule was applied to attribute
652 OTU sources for each sink (g: {abchdef}; h: {acbgedf}). Percentages on arrows indicate source
653 contributions.

654 **Fig.6:** Core microbiota network of OTUs found in cast and rhizosphere of all soils, but not
655 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 \pm 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 \pm 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
Cambisol 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 leptosol	56.7	4.7	7.5	34.4%	39.2%	27.4%	Clay soil

671

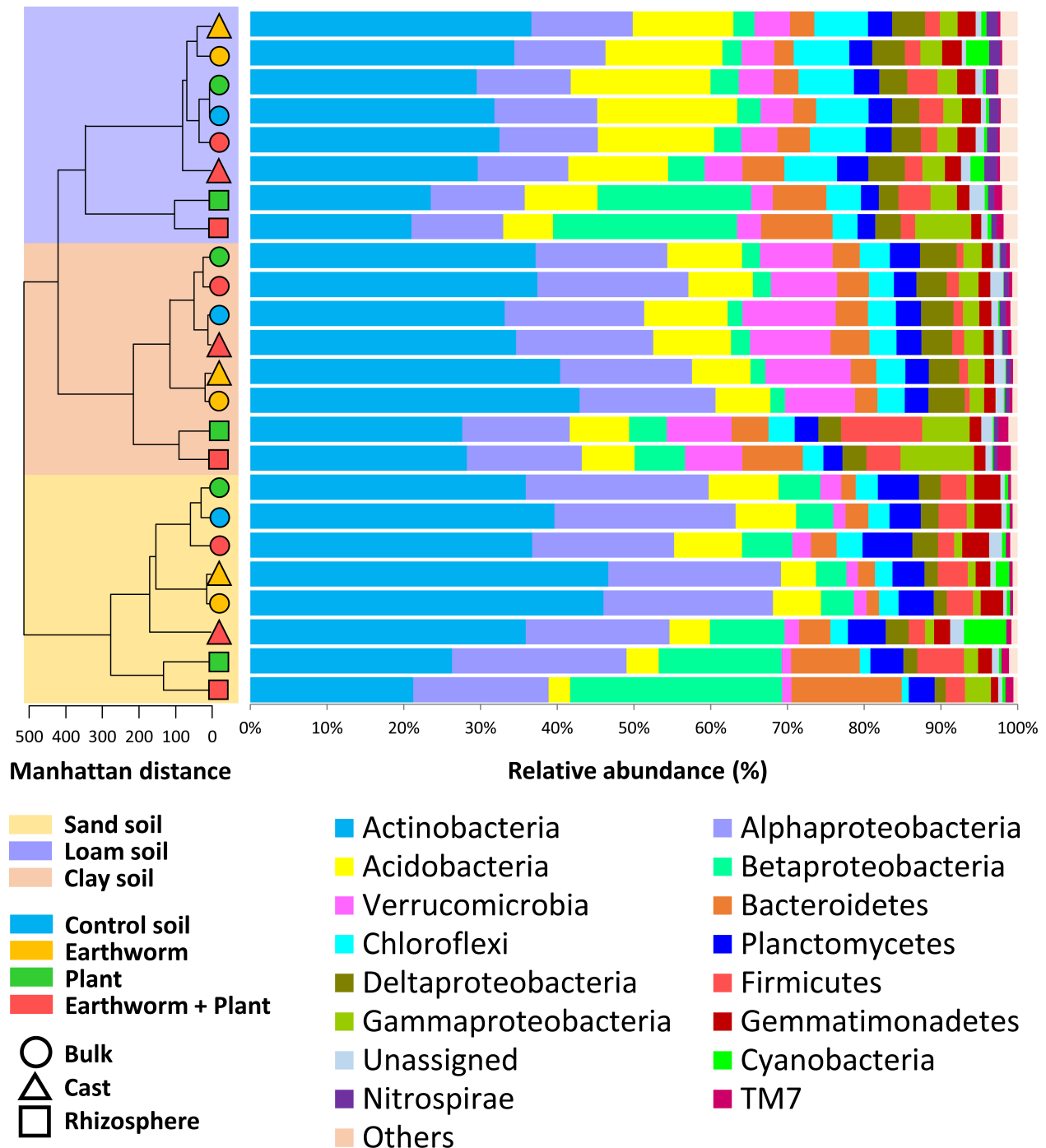
672 **Tab.2:** Effect of earthworms on barley traits at harvest in each soil. Data were collected only
673 for the treatments that had plants, with or without earthworms (green and red treatments, Fig.1).
674 Three traits were measured (rows), including height (the longest leaf length, which was always 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 (\pm 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.

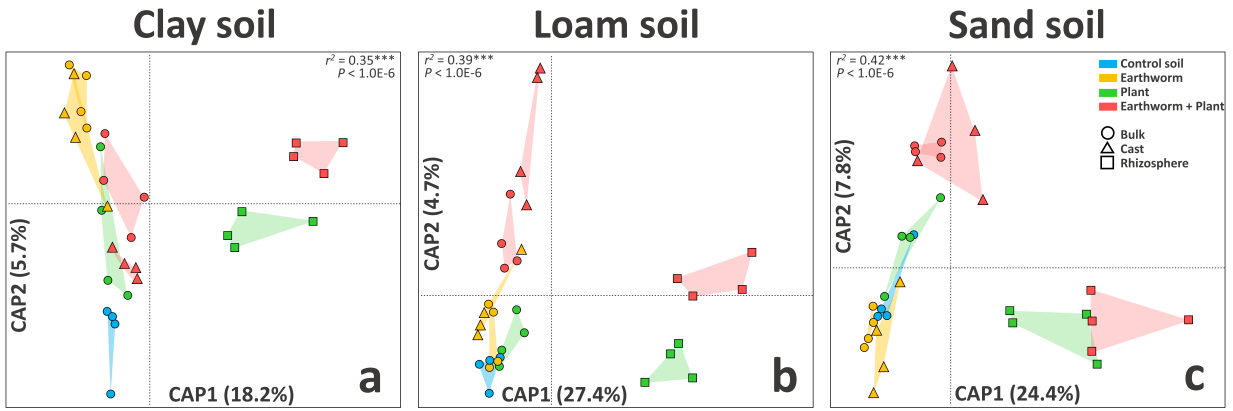
Soil	Clay		Loam		Sand	
	-	+	-	+	-	+
Height (mm)	34.80 \pm 0.26 ^b	37.10 \pm 0.8 ^a	30.30 \pm 0.51	31.10 \pm 0.6	32.10 \pm 0.56 ^b	34.3 \pm 0.44 ^a
Dry weight (g)	0.44 \pm 0.01 ^b	0.60 \pm 0.05 ^a	0.32 \pm 0.03 ^b	0.370 \pm 0.03 ^a	0.36 \pm 0.01 ^b	0.44 \pm 0.02 ^a
Surface (mm²)	47.59 \pm 2.88 ^b	54.20 \pm 2.51 ^a	36.22 \pm 2.32	35.50 \pm 2.26	41.63 \pm 1.83 ^b	47.33 \pm 2.49 ^a

680

Experimental design	Sand soil	X	Control	Earthworm	Plant	Both
	Loam soil		No macroorganisms	<i>Aporrectodea caliginosa</i>	<i>Hordeum vulgare</i> L.	<i>Aporrectodea caliginosa</i> + <i>Hordeum vulgare</i> L.
Microhabitat sampling strategy	Clay soil	X				
	Bulk					
	Cast		—		—	
Rhizosphere			—	—		

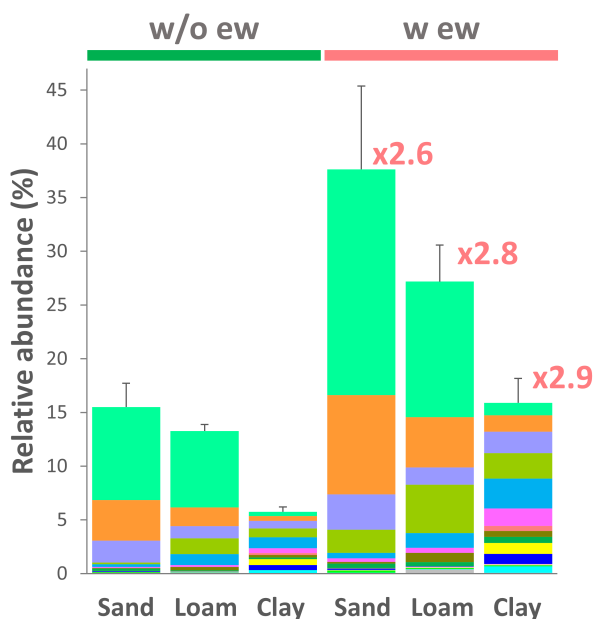
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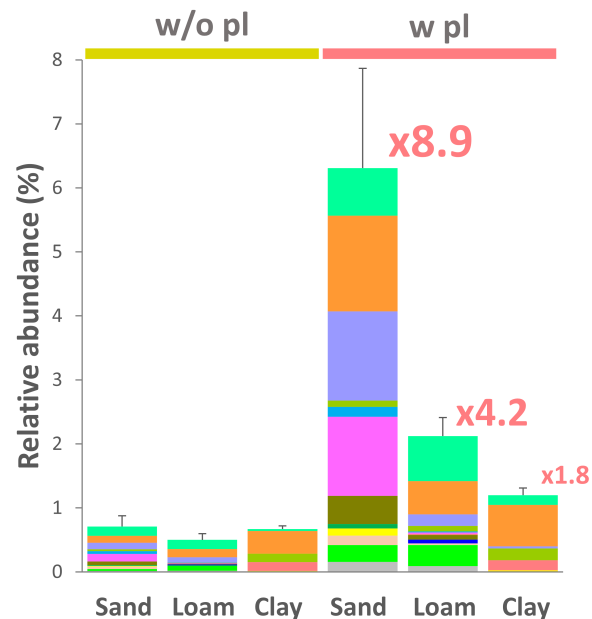


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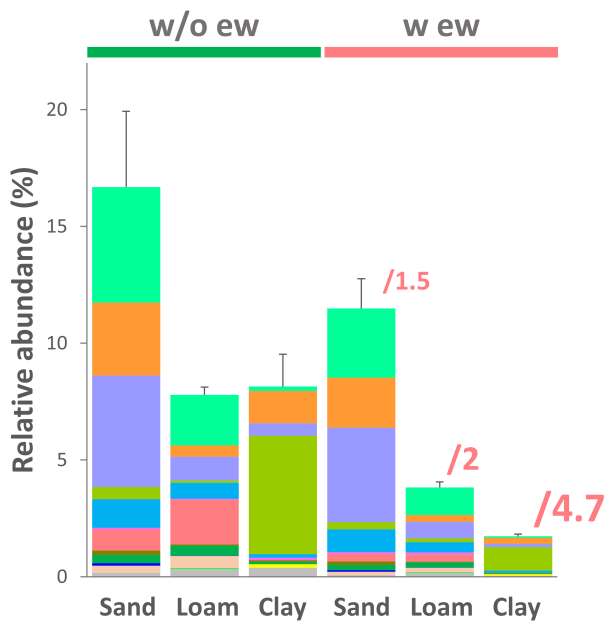
a. Rhizosphere increased OTUs



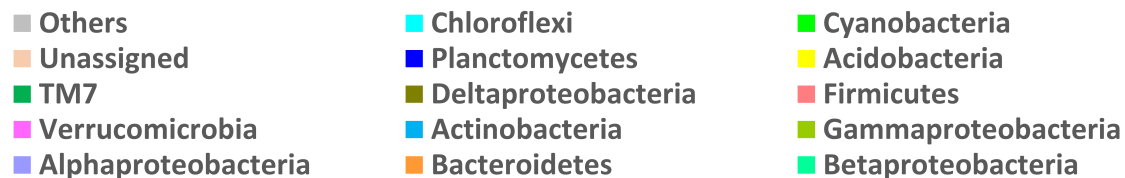
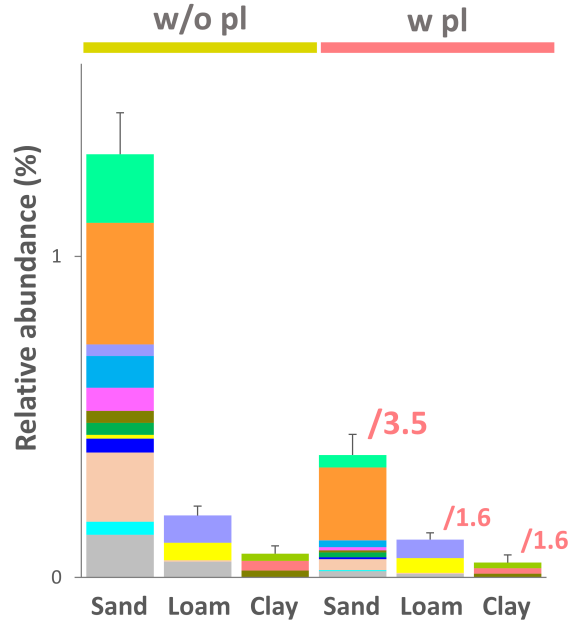
b. Casts increased OTUs

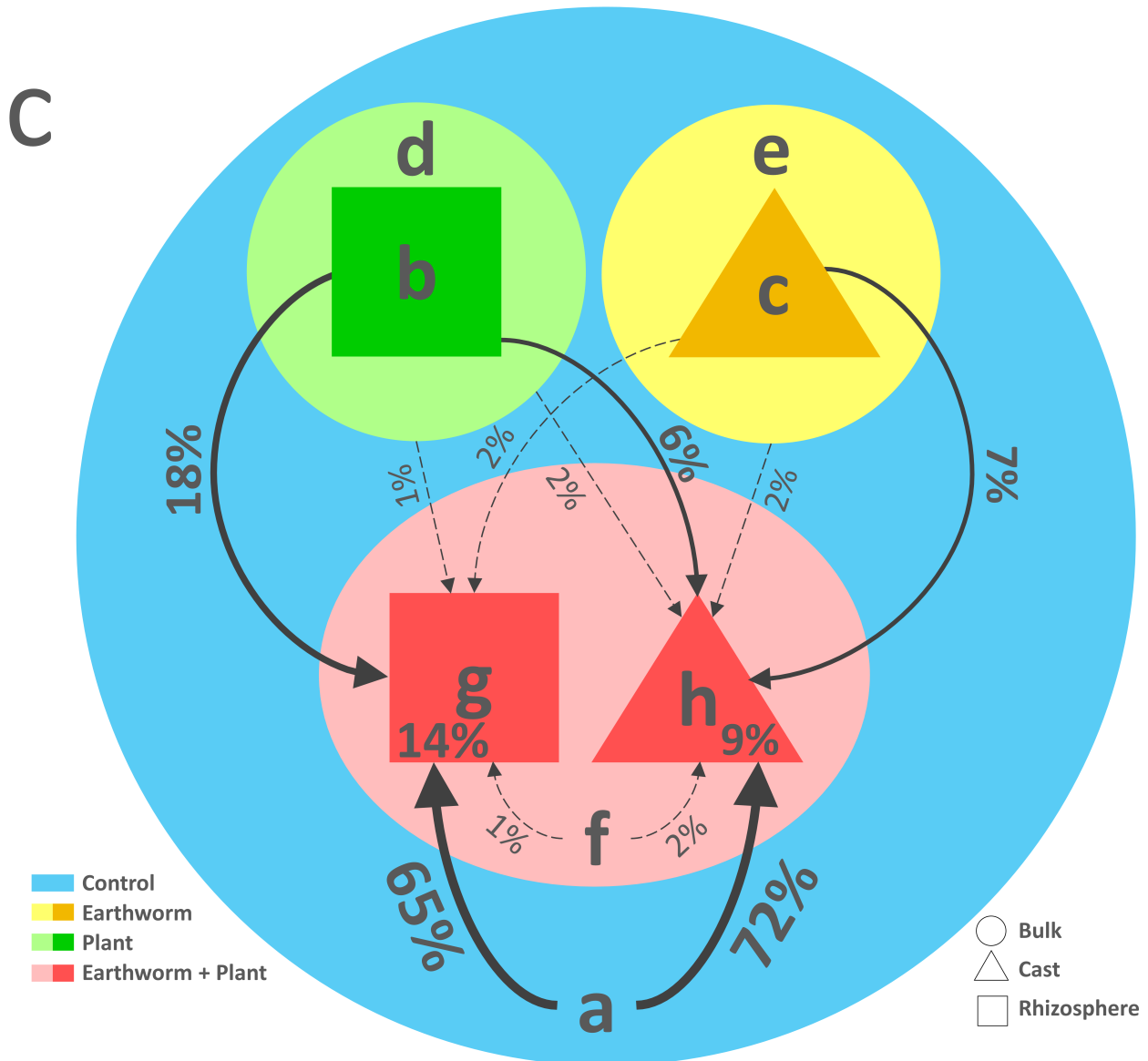
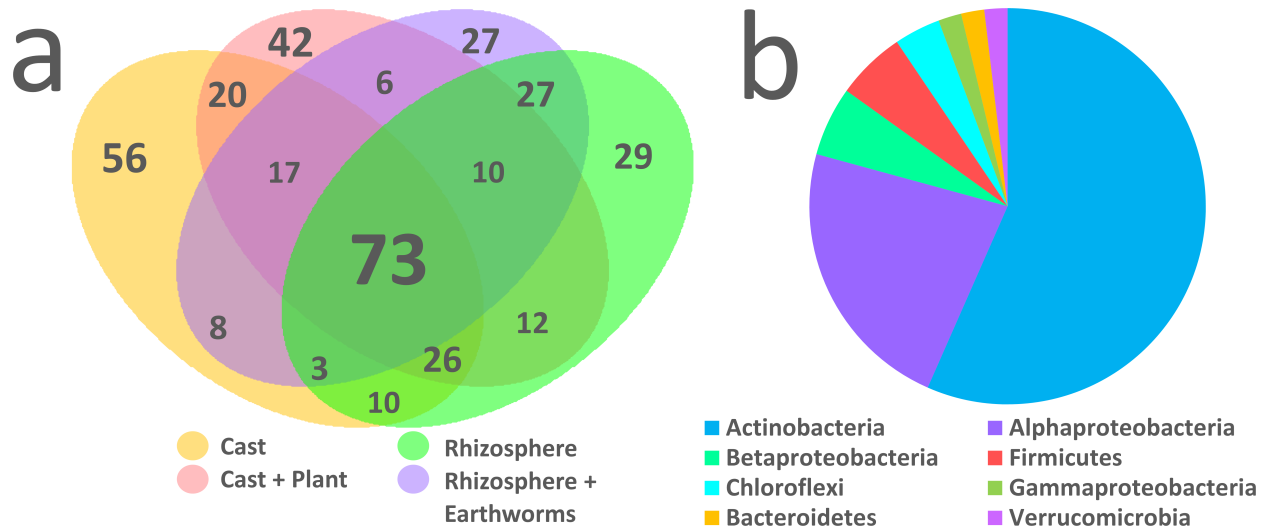


c. Rhizosphere decreased OTUs

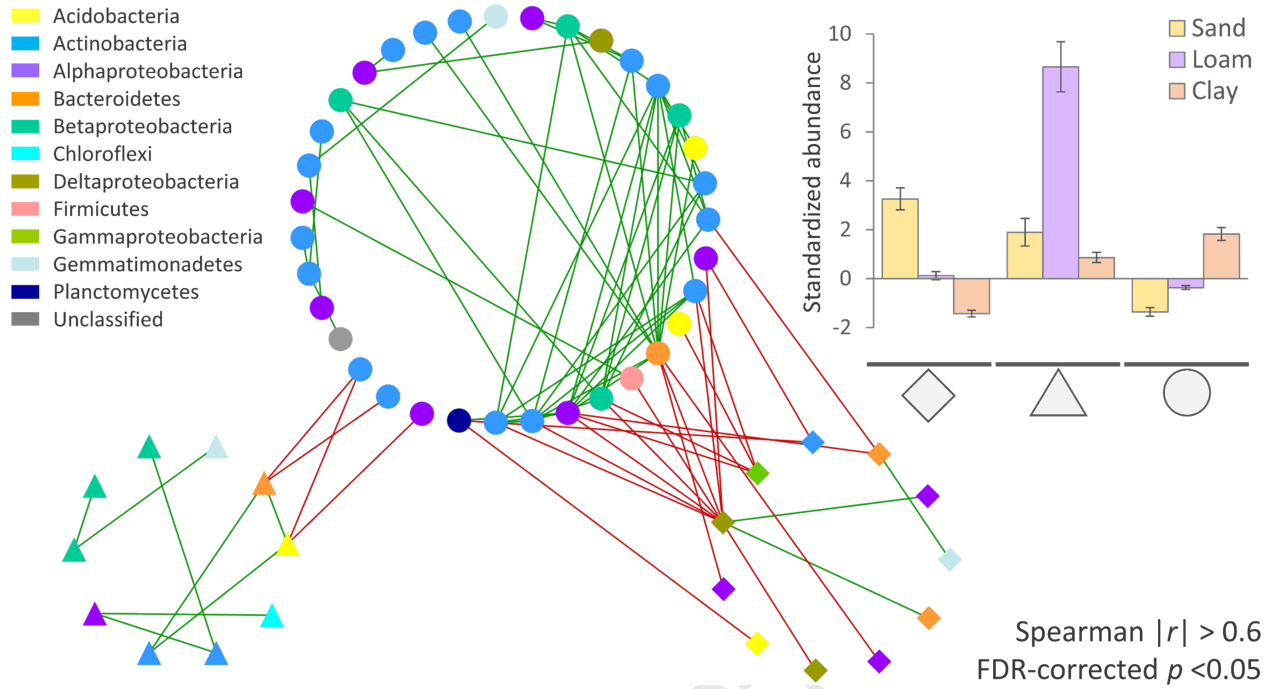


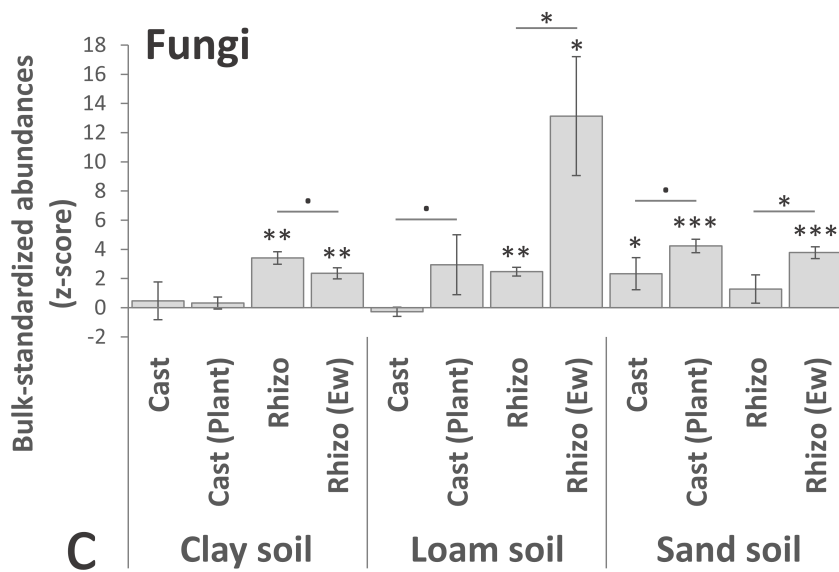
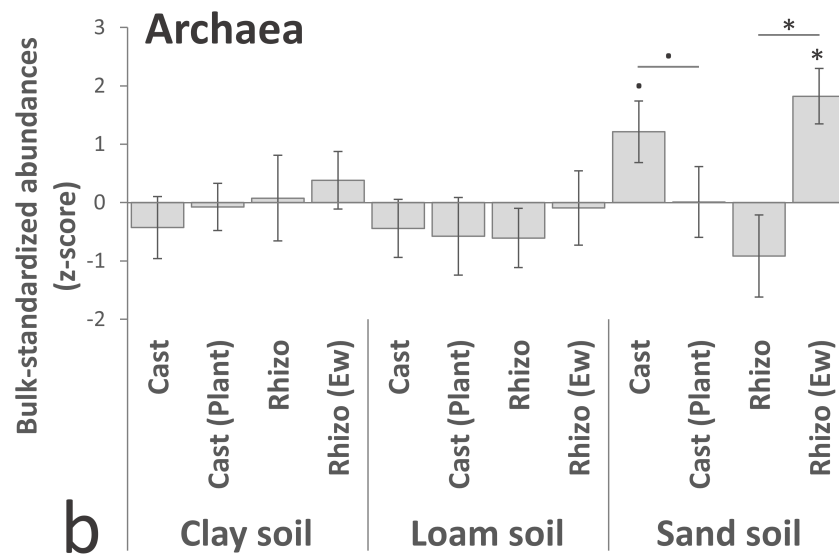
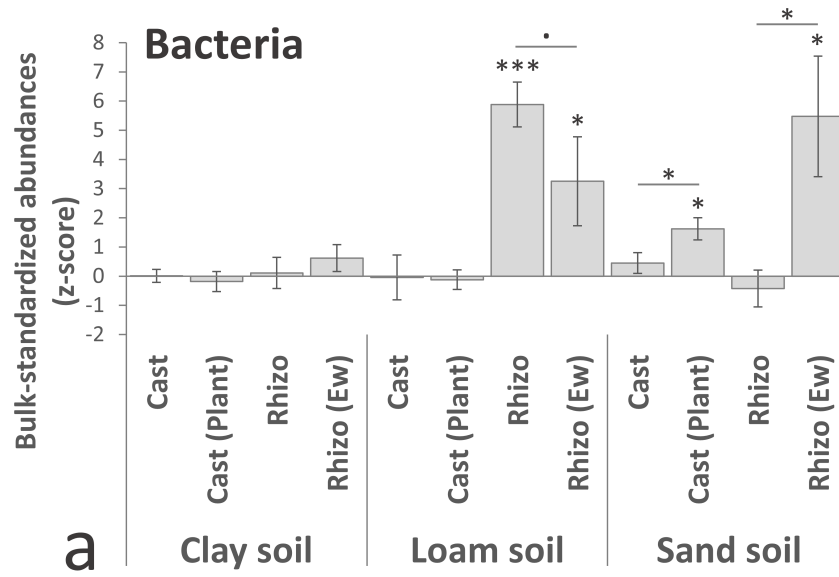
d. Casts decreased OTUs





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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: