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Microbiome dynamics during cast ageing in the earthworm *Aporrectodea caliginosa*

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ABSTRACT

Earthworm casts are hotspots of microbial activity playing an important role in the nutrient cycling of soil ecosystems. Cast ageing can be considered an ecological succession, where copiotrophic bacteria are replaced by oligotrophic bacteria as the ratio labile/recalcitrant substrates decreases. Here we use next-generation sequencing to describe the bacterial communities (microbiome) of fresh casts (day 0) from the earthworm *Aporrectodea caliginosa* and how they change over time (7, 15, 30 and 60 days) under natural conditions. We also look for the main, transitional and day-specific core microbiomes of *A. caliginosa* casts and assessed whether bacterial communities in each ageing stage are clearly distinct in their composition or can be merged into metacommunities. Fresh cast microbiomes were mainly comprised of Bacteroidetes and Proteobacteria (62% of the total sequences) and, in a lesser extent, of Acidobacteria, Actinobacteria Chloroflexi, Planctomycetes and Verrucomicrobia (36% of the total sequences). These fresh casts were richer in amplicon sequence variants (ASVs) than soil microbiomes, which also had lower abundances of Bacteroidetes but higher abundances of other bacterial phyla. As expected, copiotrophic bacteria (Alphaproteobacteria but not Bacteroidetes) significantly ($P < 0.0001$) decreased in abundance with cast ageing, while oligotrophic bacteria (Actinobacteria, Acidobacteria and Deltaproteobacteria) increased ($P < 0.03$) their proportion. This also resulted in a significant ($P < 0.05$) decrease in alpha-diversity over the first 15 days and larger differences in beta-diversity among bacterial communities of ageing casts. Despite differences in beta-diversity, microbiomes of ageing casts were grouped into two metacommunities, one comprised of younger samples collected at days 0 and 7, and another comprised of older samples collected at days 15, 30 and 60. These two metacommunities corresponded to decreased patterns in labile C and N pools in cast ageing. We found a main core microbiome comprised of eight bacterial taxa (~5% of the total sequences). Additionally, we also found transitional and day-specific core microbiomes that were richer and compositionally different from the main core microbiome. This suggests different levels of redundancy in the cast microbiomes to buffer responses to external perturbations (e.g., weather oscillations).

1. Introduction

Earthworms are important members of temperate terrestrial ecosystems where they represent the largest animal biomass component of the soil (Lavelle and Spain, 2001). Endogeic earthworm species like *Aporrectodea caliginosa* (Lumbricidae) live in non-permanent horizontal burrows in the upper soil layer, ingest large amounts of soil (Lavelle and Spain, 2001) and selectively forage organic-matter rich soil (Marhan and Scheu, 2006). Such earthworms can impact soil nutrient and microorganisms both directly (i.e., gut associated processes; GAPs) or indirectly (i.e., casts associated processes; CAPs) (Aira et al., 2003, 2005, 2010; Aira and Domínguez, 2011; Gómez-Brandón et al., 2011). Earthworm casting, mostly from endogeic earthworms, represents one of the most important processes in soil horizon turn over. In fact, in temperate zones, cast production ranges from 36 to 108 Mg ha^{-1} $year^{-1}$ (Lavelle and Spain, 2001). Hence, considering that most Lumbricidae earthworms are endogeic (Domínguez et al., 2015), earthworm casting is a key process in soil biogeochemical cycles.

Earthworm casts are biogenic structures more stable than soil macroaggregates (Lavelle and Spain, 2001). They can also persist in

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soils for a long time, depending on temperature and moisture conditions, changing their nutrient contents and microbial properties in a process known as cast ageing (Aira et al., 2005, 2010). Earthworm casts are then considered one of key contributions earthworm make to soils.

It has been shown that gut passage in endogeic earthworms (i.e. GAPs) markedly modifies microbial community structure and composition (Aira et al., 2003; Thakuria et al., 2009; Nechitaylo et al., 2010; Aira and Domínguez, 2014; Jirout and Pizl, 2016). We have also found that cast ageing (i.e., CAPs) is characterized by a continuous decline of labile C and N nutrient pools not corresponded with a decrease of microbial biomass (Aira et al., 2005, 2010) – although the structure of the microbial community does seem to change (Aira et al., 2010). Indeed, cast ageing can be considered as an endogenous heterotrophic ecological succession, characterized by fast changes, no input of external nutrients and gradual changes of substrate quality and quantity due to microbial activity (Fierer et al., 2010). It is then expected that in this type of succession labile nutrient pools support the growth of copiotrophic bacteria that, eventually, will be replaced by oligotrophic bacteria able to metabolize the remaining recalcitrant substrates in the casts (Fierer et al., 2010). However, changes in microbial communities between soil and fresh casts, as well as the composition and dynamics of these microbial communities during cast ageing are poorly understood.

Towards that aim, we have designed a field experiment to characterize the bacterial communities in casts from the earthworm *Aporrectodea caliginosa* where: i) we compare fresh cast and uningested soil microbiomes, and ii) study cast ageing from 0 to 60 days. Uningested soils and cast microbiomes were described using next-generation sequencing (Illumina MiSeq) of 16S rRNA–V4 amplicons and sequence variant analysis (dada2) and their composition, structure and core membership compared across multiple time points.

2. Materials and methods

2.1. Experimental design

Aporrectodea caliginosa (Lumbricidae) is the most abundant earthworm from Paleartic grassland regions and the most commonly found in agricultural ecosystems across temperate regions. It belongs to the endogeic earthworms, an ecological group characterized by inhabiting organo-mineral soils in which the members form an extensive horizontal network of burrows in search of soil organic matter. Although *A. caliginosa* can obstruct the burrows with its casts, this species generally egests its casts onto the soil surface (Aira et al., 2003, 2005, 2010).

We chose a field site in a grassland ecosystem at the University of Vigo (NW Spain) with a well-established population of *A. caliginosa* (population density = 136 ± 12 individuals/m^2, mean biomass = 63 ± 5 g/m^2). The site was examined carefully for earthworm activity and 50 new fresh casts deposited the day of sampling were collected in the morning. Soil was also collected from nearby grassland, sieved using a 2 mm-mesh strainer and used to fill each cell of a seed tray (54 × 28 × 15 cm). Then the tray were covered with a fine mesh (1 mm) to avoid mixing and earthworm casts were placed on top, so each cell acted as independent replicate. Seed tray was then taken back to the field site where samples were subjected to natural weather conditions during two months from late February to late April (mean temperature of 9 °C and 498 l m^{-2} of accumulated precipitation). Five cells were then randomly taken after 0, 7, 15, 30 and 60 days and casts collected. We also collected five composite bulk soil samples (i.e. free from casts) from the same field site were casts were sampled to include into the analysis.

2.2. DNA extraction, sequencing and analysis of 16S rRNA–V4 amplicons

DNA from soil and cast samples (0.25 g) was extracted using the PowerSoil DNA Isolation kit (MoBio Laboratories Inc., Carlsbad, California) according to the manufacturer's protocol. We amplified and sequenced a fragment of the 16S rRNA gene covering the V4 region (GTGCCAGCMGCCGCGGTAA forward and GGACTACHVGGGTWTCTAAT reverse) with a dual-index sequencing strategy described by Kozich et al. (2013). Sequencing was performed using the next-generation sequencing Illumina MiSeq platform at the Center for Microbial System, University of Michigan.

16S rRNA–V4 amplicon sequence variants (ASV) in each sample were inferred using DADA2 v1.8 (Callahan et al., 2016) as indicated in its online tutorial, but with modifications to suite our data (https://benjjneb.github.io/dada2/tutorial.html). Exact sequence variants provide a more accurate and reproducible description of amplicon-sequenced communities than is possible with OTUs defined at a constant level (97% or other) of sequence similarity (Callahan et al., 2017). Forward and reverse read pairs were trimmed, filtered and truncated at 230 nt and 200 nt, respectively. No ambiguous bases were allowed and each read was required to have < 2 expected errors based on their quality scores. ASVs were independently inferred from forward and reverse sequences in each sample using the run-specific error rates and then paired. Chimeras were identified in each sample and ASVs were removed if identified as chimeric in a sufficient fraction of the samples in which they were present. Taxonomic assignment was performed against the Silva v132 database using the implementation of the RDP naive Bayesian classifier available in the dada2 R package (min boot 80, Quast et al., 2013; Wang et al., 2007). We remove 15 chimeras and singleton and doubleton ASVs. A total of 414,078 sequences (mean: 13,802, SD: 4132) passed all quality filters and were assigned to ASVs (5198 and 3219 before and after rarefaction, respectively). Rarefaction curves reached saturation for all samples, which indicates that the chosen sampling depth (3308 sequences) was optimal (Sup. Fig. 1). Sequence data have been uploaded to the GenBank SRA database under accession PRJNA495452.

2.3. Statistical analysis

Samples were rarefied to the smallest sample size (3308 sequences) to remove the effect of sample size bias on community composition. We first defined the core microbiome of the ageing casts as that comprised of ASVs present in all samples. Similarly, we also defined day-specific core microbiomes as those comprised of ASVs present in samples from each time point (days 0, 7, 15, 30 and 60), and transitional core microbiomes as those comprised of ASVs present in samples from the following time-pairs: 0–7, 7–15, 15–30 and 30–60. We also looked for a transitional core microbiome from soil to 0 days casts. Day-specific and transitional core microbiome are useful to determine membership dynamics in longitudinal studies of microbial communities (Hamady and Knight, 2009).

An approximately maximum-likelihood phylogenetic tree was inferred using FastTree 2.1 (Price et al., 2010). Taxonomic alpha-diversity was estimated as the number of observed ASVs (Sobs), Shannon diversity and Chao1 richness. Phylogenetic diversity was calculated as in Faith (1992). Alpha-diversity calculations were carried out in mothur v1.39.5 (Schloss et al., 2009) 1000 times, selecting each time a random subset of 3308 sequences and given the mean value. Differences in alpha-diversity between soil and fresh casts (day 0) were tested using the *t*-test. The effect of cast ageing (days 0, 7, 15, 30 and 60) on alpha-diversity of bacterial communities from casts was analyzed using liner mixed-models (LME) analysis (nlme R package, Pinheiro et al., 2017). We set up a model with time (days) as fixed factor and the effect of time nested in each sample as a random factor to account for the non-independence of samples due to sampling over the same seed tray.

Normality of residuals and homogeneity of variance across groups was checked for each variable. The Tukey's test was used for post-hoc comparisons and the Benjamini-Hochberg FDR was used as multiple test correction method (library multcomp in R; Hothorn et al., 2008). We used the same LME models to study changes in relative mean abundance of bacterial phyla and classes during cast ageing, and of

ASVs from the core microbiome during cast ageing.

Taxonomic beta-diversity at the ASV level was estimated as the difference in bacterial taxonomic community composition between samples. This was done coupling principal coordinate analysis (PCoA) with distance matrixes that take the abundance of ASVs into account (Bray-Curtis) or not (Jaccard). Phylogenetic beta-diversity was also estimated by PCoA of weighted (considering abundance of ASVs) and unweighted unifrac matrix distances (Lozupone and Knight, 2005). All PCoAs were carried out using the phyloseq library (McMurdie and Holmes, 2013). The effect of ageing (0, 7, 15, 30 and 60 days) on both taxonomic and phylogenetic beta-diversity of bacterial communities from casts was analyzed with the same mixed models used for alpha-diversity over PCoA scores of first two axes. Differences in β-diversity between soil and fresh casts (0 days) were tested with *t*-tests over PCoA scores of first two axes.

Bacterial communities of soil and ageing casts were clustered into metacommunities using Dirichlet multinomial mixtures modelling and its fit was assessed using the Laplace approximation to the negative log model (Holmes et al., 2012; Ding and Schloss, 2014) as implemented in mothur. All analyses were performed with R 3.5 (2018) and mothur.

3. Results

3.1. Microbiome composition of fresh casts

Soil bacterial communities comprised 988 ASVs from which 140 (out of 1089 ASVs) were also present in fresh casts (0 days). These 140 soil ASVs represented 28% of the sequences of fresh casts and soil samples. Passage through the *A. caliginosa* gut significantly ($P = 0.002$) decreased the abundance of Chloroflexi (14% of the sequences), Proteobacteria (26%, $P = 0.011$), Verrucomicrobia (21%, $P = 0.007$) and Acidobacteria (10%, $P < 0.001$), and significantly increased the abundance of Bacteroidetes (355%, $P < 0.001$) (Fig. 1). Fresh casts (0 days) were dominated by Bacteroidetes and Proteobacteria (34 and 28% of the sequences) with minor contributions of Acidobacteria (12%), Verrucomicrobia (10%), Actinobacteria (7%), Planctomycetes (5%) and Chloroflexi (2%) (Fig. 1). The most abundant Proteobacteria classes were Gammaproteobacteria (16% of the sequences), Alphaproteobacteria (7%), and Deltaproteobacteria (5%) (Sup. Fig. 2). In addition, the most abundant Bacteroidetes class was Bacteroidia (33% of the sequences) (Sup. Fig. 2). The most abundant Acidobacteria classes were Acidobacteriia (1% of the sequences), SubGroup_6 (2%) and Blastocatellia SubGroup_4 (7%), whereas those for Actinobacteria were Actinobacteria (3%) and Thermoleophilia (2%) (Sup. Fig. 2). Similarly, the most abundant Planctomycetes classes were Planctomycetacia (4% of the sequences) and Phycisphaerae (1%); and Verrucomicrobiae (10%) was the most abundant class from the Verrucomicrobia phylum (Sup. Fig. 2).

3.2. Microbiome composition of ageing casts

Bacterial composition of casts changed drastically during ageing at phylum, class and ASV levels (Figs. 1 and 2, Sup. Figs. 2 and 4). Taxa exhibit different temporal patterns, some bacterial phyla [Proteobacteria ($F_{4,16} = 41.04$, $P < 0.0001$), Chloroflexi ($F_{4,16} = 13.45$, $P < 0.0001$) and Actinobacteria ($F_{4,16} = 3.47$, $P = 0.032$)] and classes [Alphaproteobacteria ($F_{4,16} = 44.03$, $P < 0.0001$), Deltaproteobacteria ($F_{4,16} = 21.02$, $P < 0.0001$), Gammaprotebacteria ($F_{4,16} = 20.15$, $P < 0.0001$), KD4–96 ($F_{4,16} = 11.11$, $P < 0.001$), Planctomycetacia ($F_{4,16} = 20.03$, $P < 0.0001$) and Thermoleophilia ($F_{4,16} = 8.10$, $P < 0.001$)] showed initial increases in mean proportion followed by a continuous decrease from day 15 to day 60 (Fig. 1, Sup Fig. 2); other phyla [Acidobacteria ($F_{4,16} = 13.86$, $P < 0.0001$) and Verrucomicrobia ($F_{4,16} = 35.14$, $P < 0.0001$)] and classes [Acidobacteriia ($F_{4,16} = 4.41$, $P = 0.013$), SubGroup_6 ($F_{4,16} = 24.25$, $P < 0.0001$) and Verrucomicrobiae ($F_{4,16} = 35.14$, $P < 0.001$)] initially decreased (days 7 and 15) to later recover their former abundances (Fig. 1 and Sup Fig. 2); two bacterial classes [Blastocatellia SubGroup_4 ($F_{4,16} = 22.07$, $P < 0.0001$) and Phycisphaerae ($F_{4,16} = 3.09$, $P = 0.045$)] continuously increased during cast ageing (Sup Fig. 2); and, finally, some phyla (Actinobacteria and Bacteroidetes) and class (Bacteroidia) did not change with ageing (Fig. 1, Sup Fig. 2).

3.3. Microbiome diversity and metacommunity assembly of ageing casts

Despite a decrease in the relative abundance of several bacterial phyla between soil and fresh cast samples, bacterial communities of cast were taxonomically richer in ASVs than those of soil (alpha-diversity, Fig. 2a), although they did not differ in taxonomic and phylogenetic diversity (alpha-diversity, Sup. Fig. 3). Soil and fresh cast microbiomes were clearly different in PCoA 1 and PCoA 2 for all distance measures (beta-diversity, $P < 0.0001$ for all PCoA axes and distances, Fig. 2b and Sup. Fig. 4). Cast ageing produced a significant decrease in alpha-diversity through time for taxonomic richness (ASV richness, $F_{4,16} = 3.12$, $P = 0.044$; Fig. 2a), as well as for Shannon diversity ($F_{4,16} = 6.71$, $P = 0.002$) and phylogenetic diversity ($F_{4,16} = 31.94$, $P < 0.0001$); but not for Chao1 richness ($F_{4,16} = 2.94$, $P = 0.053$; Sup. Fig. 3). The phylogenetic and taxonomic composition of casts microbiomes also changed drastically with ageing (beta-diversity, Fig. 2b and Sup. Fig. 4). Thus, bacterial communities of fresh and day 7 casts always differed in PCoA 2 for all distances except weighted Unifrac, whereas they only changed in PCoA 1 for unweighted Unifrac distances (Fig. 2b, Sup. Fig. 4). Similarly, cast microbiomes of days 0 and 7 clearly differed from those of days 15, 30 and 60 (see PCoAs in Fig. 2b and Sup. Fig. 4).

All PCoA plots suggest that soil and early (0 and 7 days) and late (15 to 60 days) casts comprise three different bacterial metacommunities. However, DMM analysis identified only two bacterial metacommunities during cast ageing (Fig. 3, insert). These bacterial metacommunities are represented by a group of relative abundant profiles of different ASVs (Fig. 3). A close examination of the 20 most important ASVs, which accounted for 18% of the difference in fit between 1 and 2 metacommunities for our data, showed that in the metacommunity type 1 (soil, days 0 and 7) five ASVs, classified as *Flavobacterium* (2 ASVs), *Cd.* Udaeobacter (1 ASV), env.OPS_17 (1 ASV) and Acidobacteria Subgroup_6 (1 ASV) were overrepresented (Fig. 3). On the other hand, the metacommunity type 2 (days 15, 30 and 60) was characterized by the remaining 16 ASVs, from which the most abundant were classified as *Massilia* (3 ASVs), *Adhaeribacter* (2 ASVs), *Arenimonas* (1 ASV) and *Brevundimonas* (1 ASV). All these ASVs were underrepresented or absent from metacommunity type 1 (Fig. 3).

3.4. A core microbiome for bacterial communities of ageing casts

Despite the marked differences among cast microbiomes, we found 8 ASVs present in all cast samples (0.2–0.3% of the total ASVs, but 4.9–5.3% of all the sequences in the rarefied dataset), which constitute the core microbiome of the *A. caliginosa* casts (Fig. 4). Only ASV17-*Caulobacter* was exclusive to casts, the other seven ASVs were also present in soil samples. From those seven ASVs, only four differed significantly between soil and day 0 casts (ASV14-Blastocatellaceae, ASV22-*Ferruginibacter*, ASV6-*Caenimonas* and ASV58-*Cellulomonas;* t.test, $P < 0.05$ for all).

Only six ASVs showed a significant change in their relative mean abundance during cast ageing (Fig. 4). Those ASVs belonged to the phyla Proteobacteria, Bacteroidetes, Actinobacteria and Acidobacteria. We also found day-specific core microbiomes for each sampling date that were richer and compositionally different than the core microbiome. Those day-specific core microbiomes were composed by 105 (day 0), 24 (day 7), 41 (day 15), 20 (day 30) and 40 (day 60) ASVs each after removing the ASVs present in the main core microbiome and soil

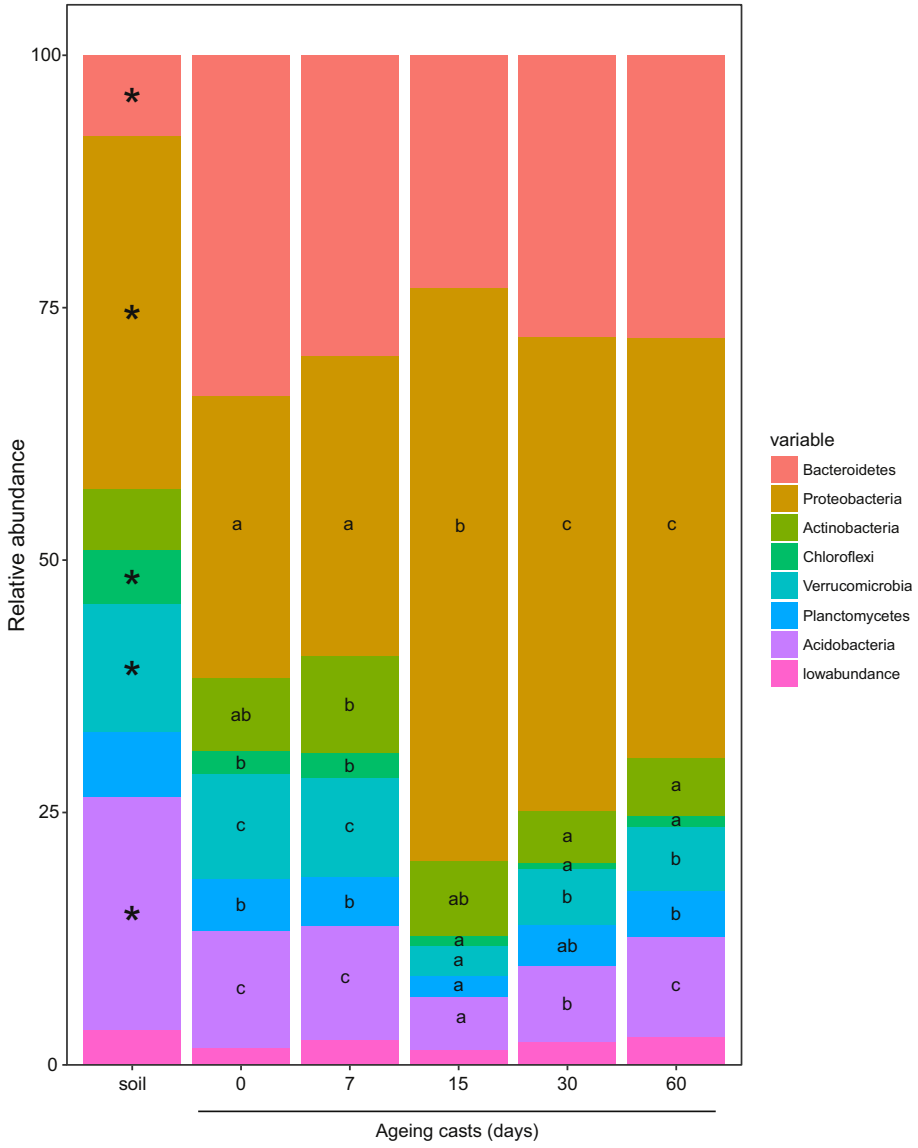

Fig. 1. Changes in microbiome composition (phylum level) during cast ageing in the earthworm *Aporrectodea caliginosa*. Significant differences (*t*-test) between soil and 0 days casts are indicated with an asterisk. Different letters for each bacterial phyla indicate significant differences between ageing times (Tukey HSD test, FDR corrected).

samples (Sup. Table 1). These five core microbiomes were also dominated by a set of bacterial phyla of variable abundances depending on sampling date and did not show a clear trend across taxa. Acidobacteria, for example, decreased its contribution to each core microbiome through time, while Bacteroidetes and Proteobacteria maintained steady contributions (Sup. Table 1). Moreover, there were also five transitional core microbiomes between sampling dates: soil-day 0, days 0–7, 7–15, 15–30 and 30–60; they comprised 5, 16, 3, 9 and 15 ASVs, respectively (Sup. Table 2). Most of the ASVs from transitional cores were included in the phyla Bacteroidetes and Proteobacteria, and only 3 ASVs changed their relative abundance between sampling dates within time-pairs (Supplementary Table 2). Moreover, some of these ASVs were shared among contiguous transitional cores (Sup. Table 2).

4. Discussion

4.1. Microbiome composition of fresh casts

We found that bacterial communities of fresh casts of the earthworm *A. caliginosa* were mainly composed (62% of the sequences) by ASVs of the phyla Bacteroidetes and Proteobacteria, with other four bacterial phyla (Acidobacteria, Actinobacteria, Chloroflexy, Planctomycetes and Verrucomicrobia) having relatively minor contributions (36%). Our results partially agree with those reported by Nechitaylo et al. (2010) for casts of *A. caliginosa*. We found a similar composition at phylum level, but with different relative abundances; Proteobacteria (52% of the sequences), Actinobacteria (24%) and Chloroflexi (8%) showed higher abundances in *A. caliginosa*, whereas Acidobacteria (8%) showed lower relative abundance. The composition of bacterial phyla of casts from *A. caliginosa* was relatively similar to that of other epigeic earthworms, *Lumbricus rubellus* (Knapp et al., 2009) and *Eisenia andrei* (Aira et al., 2015, 2016), and another anecic earthworm, *L. terrestris* (Wüst et al., 2011). Cast microbiomes from these three earthworm species showed different proportions of OTUs (not ASVs) belonging to the phyla Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Proteobacteria, Planctomycetes and Verrucomicrobia, among others. Cast microbiomes from *E. andrei,* L. *rubellus* and *L. terretris* showed higher proportions of Proteobacteria (> 30%) and Actinobacteria, Acidobacteria, Bacteroidetes and Firmicutes (17–30%), than those of any other bacterial phyla. This partially agrees with our results here for *A.*

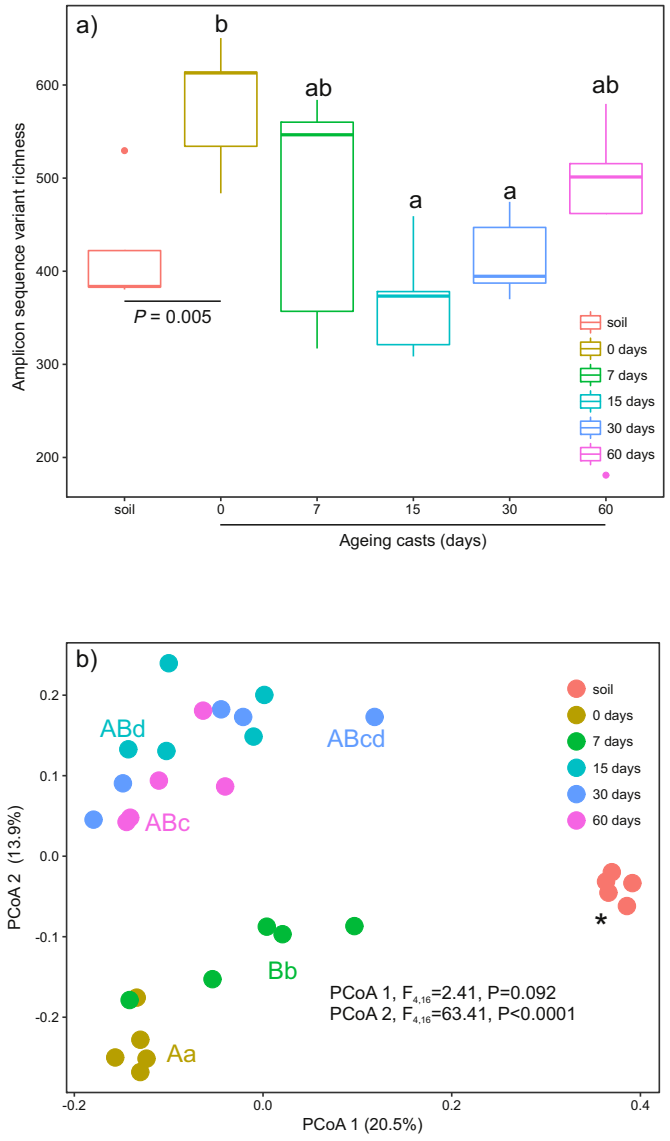

Fig. 2. Changes in microbiome diversity during cast ageing in the earthworm *Aporrectodea caliginosa*. a) alpha-diversity estimated as observed amplicon sequence variant (ASV) richness, and b) beta-diversity measured as principal coordinate analysis of unweighted Unifrac distances. Significant differences between soil and 0 days casts are indicated with an asterisk in the PCoA plot (means were separated with t-test, $P < 0.0001$ for the two PCoA axes). Different letters indicate significant differences between ageing times in the alpha-diversity plot (Tukey HSD test, FDR corrected). Different capital and lowercase letters indicated significant differences between treatments during casts ageing in PCoA 1 and PCoA 2 respectively in the beta-diversity plot (Tukey HSD test, FDR corrected).

caliginosa casts, since Firmicutes were almost absent from *A. caliginosa* casts (1.4% of the sequences). Moreover, bacterial communities of casts were clearly different from those of soil, and more diverse, which agrees with previous results for the same earthworm species (Thakuria et al., 2009; Nechitaylo et al., 2010).

4.2. Microbiome composition of ageing casts

Cast ageing did not impact all bacterial phyla the same way; thus, some phyla (Acidobacteria, Planctomycetes and Verrucomicrobia) initially decreased to lately recover their initial values, while others (Actinobacteria, Chloroflexy and Proteobacteria) showed an initial increase followed by a continuous decrease towards the end of the ageing process. These changes in microbiome composition across ageing cast is likely related to the trophic stage (oligotrophic vs copiotrophic) of their members. Oligotrophs and copiotrophs differ in their growth strategies under nutrient rich conditions (*r* compared with *k*) and the C substrates that can metabolize (labile and recalcitrant for oligotrophs and copiotrophs, respectively) (Fierer et al., 2007; Bastian et al., 2009; Trivedi et al., 2013). Hence, copiotrophic bacteria will be more abundant in initial stages of the microbial succession and will lead the decomposition of substrates rich in organic matter (like earthworm casts) (Aira et al., 2003, 2005); they will be later replaced by oligotrophic bacteria when the proportion of recalcitrant nutrients increase (Fierer et al., 2007; Jackson, 2003). In agreement with this hypothesis, we have seen in our study higher relative abundances of copiotrophic bacteria (Alphaproteobacteria, and Bacteroidetes) in initial stages (days 0 to 15) of the cast ageing process, whereas oligotrophic bacteria (Actinobacteria, Acidobacteria and Deltaproteobacteria) partially dominated the cast microbiomes in the last stages (days 30 and 60).

4.3. Microbiome diversity and metacommunity assembly of ageing casts

Changes in microbiome composition during cast ageing drastically affected bacterial alpha-diversity, which significantly dropped after 15 days, remaining relatively constant until the end of the ageing process. Since alpha-diversity (taxonomic and phylogenetic) is related to ecosystem function (Cadotte et al., 2008), this has important consequences for organic matter decomposition, a key process in terrestrial ecosystems. For example, this fall in alpha-diversity could be related to the continuous decline observed in microbial activity in *A. caliginosa* during cast ageing (Aira et al., 2005, 2010). Interestingly, bacterial communities of casts strongly differ at taxonomic and phylogenetic levels during ageing, forming three groups or metacommunities of samples (day 0, day 7 and days 15–30–60). This grouping is supported by quantitative (Bray-Curtis) and qualitative beta-diversity indices (Jaccard). Quantitative changes would imply differences in abundance of ASVs during ageing; such changes may be related to temporal factors (Lozupone et al., 2007) like nutrient depletion associated to cast ageing (Aira et al., 2005). On the other hand, qualitative changes would imply that specific ASVs thrive exclusively in each of the analyzed sampling time points, and that may be due to uneven microbial growth or temporal turnover during bacterial succession (Lozupone et al., 2007). These same differences are also seen when phylogenetic information is considered (unifrac distances), indicating that the microbiomes of ageing casts are phylogenetically distant. This last result also suggests that bacteria from each time point have developed specific adaptations to live in an environment that is continuously diminishing its nutrient composition from labile to recalcitrant nutrients.

Together, the decline in alpha-diversity and the grouping of samples in initial, mid and final stages of cast ageing found in the beta-diversity analyses, suggest that bacterial communities of ageing casts in *A. caliginosa* could be grouped in at least three metacommunities (day 0, day 7 and days 15–30-60). Our DMM analysis, however, suggested only two metacommunities (days 0–7 and days 15–30-60). The latter partitioning could be related to marked changes observed in nutrient pools after 15 days of cast ageing were some nutrients are almost depleted (N-NH_4^+) whereas others started to increase (N-NO_3^-, dissolved organic C and N; Aira et al., 2005). Surprisingly, despite a drastic difference in composition (bacterial phyla and classes) and diversity, soil samples were also included in the days 0–7 DMM metacommunity.

4.4. A core microbiome for bacterial communities of ageing casts

Our analysis of 16S rRNA–V4 amplicons found a set of 8 ASVs in all analyzed samples that constitute the core microbiome of ageing casts in *A. caliginosa* (Shade and Handelsman, 2012). The only bacterial ASV of core microbiome that was not present in soil samples was ASV17, *Caulobacter*. There is only a study, in which *Caulobacter* was found in

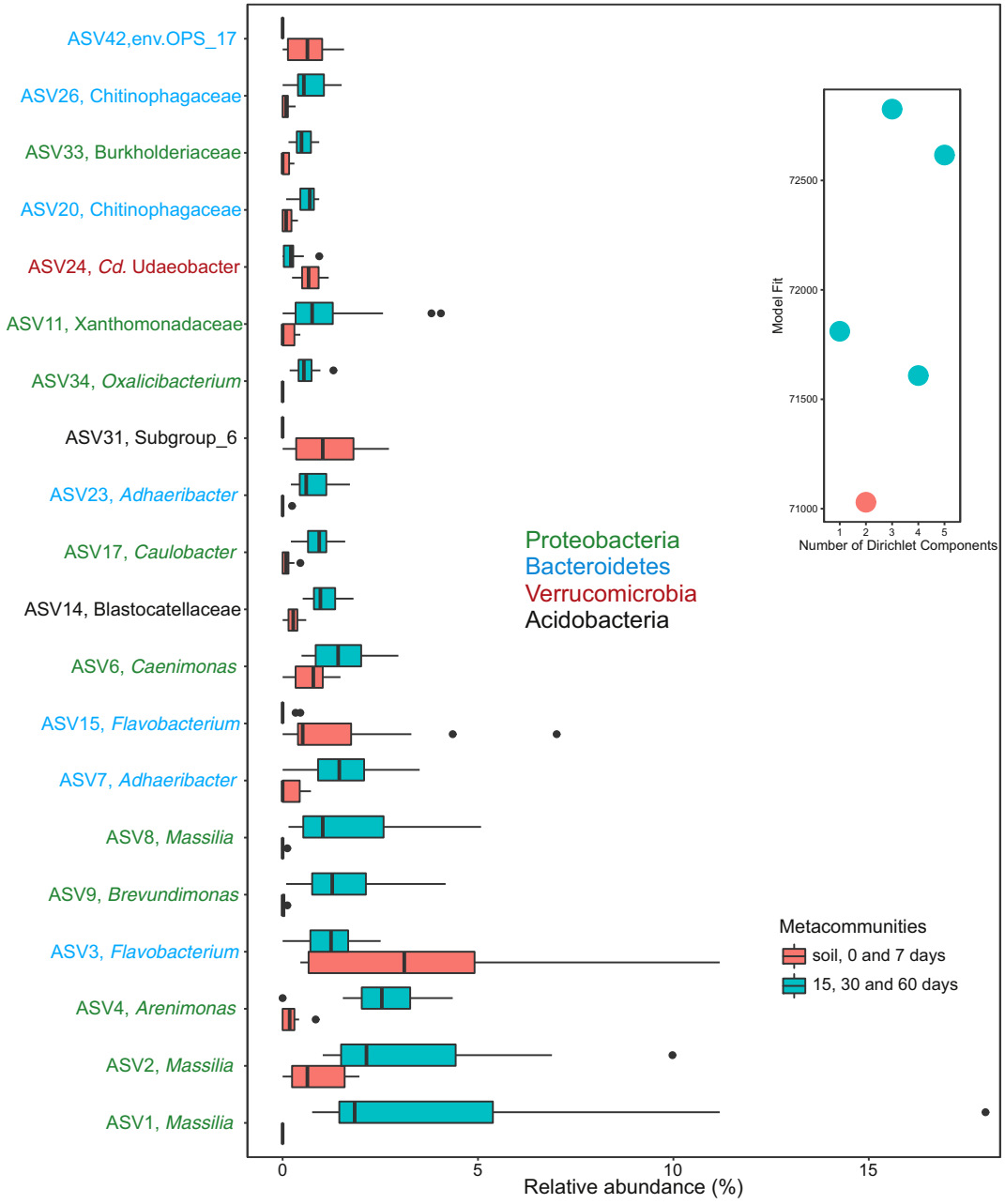

Fig. 3. Relative abundance of the most abundant ASVs in the samples assigned to each of the two metacommunity types found in ageing casts of the earthworm *Aporrectodea caliginosa*: metacommunity type 1 (red) and 2 (blue) correspond to soil and days 0–7, and days 15–30-60. The insert represents the support for two metacommunity types when applying Dirichlet multinomial mixture models. ASVs are sorted in decreased order of importance from top to bottom. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

soil, but not in *A. caliginosa* casts (Liu et al., 2011). Differences with our data could arise from the fact that they analyzed the active part of bacterial community and to the lack of resolution of T-RFLP used in that study.

Moreover, we found a core microbiome for each sampling date, and we also found transitional cores between each pair of contiguous sampling dates. Those transitional cores were larger for days 0–7 and 30–60s than for days 7–15 and 15–30, which also reinforces the result of the DMM analysis. Although the core microbiome was small (only 8 ASVs), it accounted for ~5% of all the sequences. These ASVs are likely critical for the optimal metabolic performance of cast microbiomes in *A. caliginosa* (Shade and Handelsman, 2012). This core microbiome did not show any degree of phylogenetic redundancy below the phylum level, since ASVs did not share their taxonomic classification. This lack of redundancy could be critical to buffer responses to external perturbations (Yachi and Loreau, 1999), such as those associated to weather changes (strong rainfalls, sudden temperature oscillations). Transitional and day-specific core microbiomes, however, showed a higher degree of phylogenetic redundancy, reinforcing this same hypothesis at shorter time-scales. This is also supported by increased similarity in bacterial communities with time and by metacommunity assembly.

5. Conclusions

Cast microbiomes from the earthworm *A. caliginosa* were diverse and mainly composed by members of the phyla Bacteroidetes and Proteobacteria. We found that bacterial phyla changed through time according to their trophic characteristics (from copiotrophs to

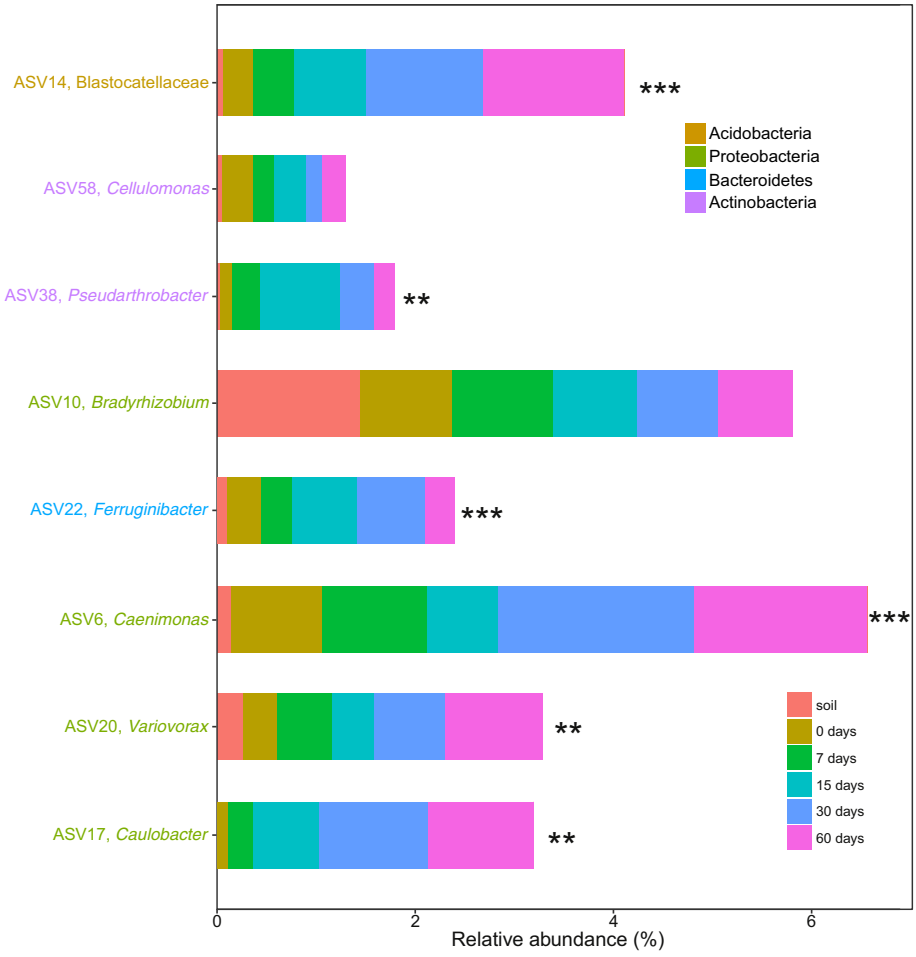

Fig. 4. Changes in relative abundance of ASVs from the core microbiome of casts of the earthworm *Aporrectodea caliginosa* during ageing. Relative abundance (%) and taxonomy (phylum and genus or most inclusive taxonomy found) of each ASVs that appeared in all samples. Six ASVs (ASV10, ASV14, ASV38, ASV6, ASV20 and ASV22) were also present in the soil samples. Significance of ANOVA tests across time points are indicated with * (** = $P < 0.01$ and *** = $P < 0.001$).

oligotrophs), as predicted in a succession system like the endogenous heterotrophic earthworm cast ageing system. Cast ageing diminished alpha-diversity, which resulted in increased differences in community structure between bacterial communities of young (0 and 7 days) and old casts (15 to 60 days). Interestingly, we found that bacterial communities across time points were grouped into two metacommunities including the same young and old casts above, in agreement with previous results describing the nutrient dynamics of ageing casts in *A. caliginosa* (Aira et al., 2005, 2010).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2019.03.019.

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