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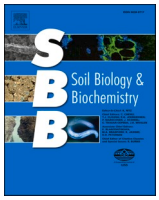

Legacy effects of earthworms on soil microbial abundance, diversity, and community dynamics

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ABSTRACT

The earthworm species *Lumbricus terrestris* L. feeds on plant litter mixed with surrounding soil. Here, we analyzed with a mesocosm approach and soil incubations how that activity and subsequent ageing of casts (feces) affects the abundance and diversity of the soil microbiome. Earthworms were fed either with straw of sainfoin (SA, *Onobrychis viciifolia;* C/N ratio 22) or winter wheat (WW, *Triticum aestivum*, C/N ratio 101). The gut transit increased the abundances of bacteria and fungi, but reduced archaea. As indicated at the DNA and RNA level, main beneficiaries of the facilitated access to nutrients were members of Bacteroidota, especially Flavobacteriales with an estimated generation time of only 2 h. While Alphaproteobacteria were reduced, Gammaproteobacteria also increased in abundance and activity. SA was more nutritious for *L. terrestris*, and supported a higher bacterial abundance, probably because more N was available for growth and denitrification. During cast ageing, prokaryotic community compositions became increasingly similar to bulk soil communities. However, they remained distinguishable even after 168 d, suggesting that effects can last beyond a vegetation period. Dry-wet conditions preserved these differences better than continuous moisture. During ageing, more complex prokaryotic networks were detected with WW and dry-wet conditions. Thus, N and water limitations appeared to enhance cooperation rather than competition between the prokaryotes. Overall, this study demonstrates that earthworm soil interactions strongly affect the diversity and temporal dynamics of the soil microbiome. Legacy effects of earthworm activities should thus be kept in mind when investigating the environmental variation of soil microbiomes.

1. Introduction

Earthworms are widely abundant soil organisms which contribute with their feeding and burrowing activity to the function of terrestrial ecosystems (Blouin et al., 2013). Anecic earthworms, like *Lumbricus terrestris* L., create vertical burrows extending up to 2-m deep into the soils. They primarily feed on plant litter which they mix with mineral soil particles, and during the gut passage the organic substrates are mixed with earthworm mucus, transformed and decomposed (Lavelle et al., 1995). Upon egestion, they use part of their fecal material to stabilize their burrow walls (Rogasik et al., 2014), and deposit other parts as cast material onto the soil surface. Compared to the surrounding bulk soil, these casts are typically enriched in organic substances and become more fertile than the surrounding bulk soils (Van Groningen et al., 2019). Furthermore, the earthworm gut passage also improves soil structure by promoting soil aggregation and macropore formation (Zhang and Schrader, 1993; Marashi and Scullion, 2003). While typically earthworm activities are not exploited in agricultural cropping systems founded on soil ploughing, non-tillage production systems can take advantage of these earthworm activities, thereby combating e.g. soil erosion and compaction (Tebrügge and Düring, 1999; Capowiez et al., 2012).

Once the ingested mixture of organic material and mineral soil enters the earthworm, it is transferred through a relatively straight, tube-like system (Drake and Horn, 2007). The host secreted mucus allows the smooth transport of the ingested material along the alimentary canal (Tiunov and Scheu, 1999). After mechanical grinding, the ingested substrates are mixed with intestinal mucus in the foregut and then subjected to digestive exoenzymes secreted by the host and the gut microbiome, including e.g. cellulases, proteases, chitinases, lipases (Urbasek and Chalupsky, 1991). It has been estimated that the average gut transit time of *L. terrestris* is 12 h and that an average single worm can produce approx. 0.7 g feces (cast) per day (Taylor and Taylor, 2014).

Microbial communities ingested with the soil material apparently

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form the major part of the gut microbiome of *L. terrestris* (Egert et al., 2004; Drake and Horn, 2007). During the gut passage the soil microbiome is subjected to specific microhabitat conditions, which may support or inhibit growth of specific taxa (Lemtiri et al., 2014; Medina-Sauza et al., 2019). Favorable conditions would exist e.g. for microorganisms capable of utilizing carbon and energy sources released by enzymatic activities from the ingested organic material, thereby supporting their growth. Due to oxygen depletion, denitrifying microorganisms gain a selective advantage and are enriched (Drake and Horn, 2007). As a result, soil nitrogen can be lost as N_2 and N_2O gas (Drake and Horn, 2006; Horn et al., 2006b; Schlatter et al., 2019). On the other hand, the gut passage in *Lumbricus* may reduce the prevalence of some taxa, as e.g. shown for ingested cells or *Escherichia coli* (Pedersen and Hendriksen, 1993; Thimm et al., 2001).

Compositional changes of soil microbial communities during earthworm gut passages, including fungi, bacteria and protists, have been demonstrated in several studies (Cai et al., 2002; Nechitaylo et al., 2010; Gong et al., 2018; Buivydaite et al., 2023; Sofo et al., 2023). Sequencing of 16S rRNA gene PCR amplicons indicated that the gut passage of *L. terrestris* favors copiotrophic bacteria, as e.g. represented in the phyla Proteobacteria, Actinobacteria or Bacteroidetes, while the abundance of suspected oligotrophs, e.g. indicated by phyla like Acidobacteria or Gemmatimonadetes was reduced (Nechitaylo et al., 2010; Shakhnazarova et al., 2021). It should be noted that these results were based on compositional analyses with the limitation that growth and death of particular microbial community members cannot be differentiated (Gloor et al., 2017). Combined with quantitative PCR, however, compositional analyses allow to distinguish both processes (Blazewicz et al., 2014) and should thus improve our understanding how earthworms affect the soil microbiome (Cai et al., 2020).

Once deposited on the soil surface, earthworm casts start an ageing process during which organic nutrients are depleted by leaching and microbial metabolism (Tiunov and Scheu, 2000a; Vidal et al., 2019). Along with the decomposition of the organic constituents and the successional release of different carbon and energy sources, cast inhabiting microbial communities are changeable (Scullion et al., 2003; Aira et al., 2019). The physicochemical structures of casts are in fact quite stable and can persist for months and years (Marashi and Scullion, 2003; Vidal et al., 2019), also preserving a different fungal community as compared to the surrounding soil even after 100-d of ageing (Tiunov and Scheu, 2000b). Considering the large amounts of soil which can be processed by earthworms under field conditions (Blouin et al., 2013), the actual composition of soil microbiomes may thus be much stronger affected by earthworm legacy effects than commonly acknowledged.

The objective of this study was to characterize the impact of earthworm feeding and casting on the abundance of the major soil microbial groups (bacteria, archaea, fungi) and the composition of the prokaryotic soil microbiome. Microbiomes were analyzed by quantitative PCR-based detection of bacterial and archaeal 16S rRNA genes and fungal ITS1 sequences. The composition of the prokaryotic microbiome was assessed by sequencing partial 16S rRNA genes and, in addition, to emphasize the detection of metabolically active community members, in parallel at the 16S rRNA level (Nechitaylo et al., 2010; Wüst et al., 2016). Despite some limitations of using rRNA as an activity marker, connected to rRNA contents that may occur in some dormant cells (Sukenik et al., 2012) or biases due to copy number differences between taxa (Wang et al., 2013), the usefulness of this approach in soil ecology has been demonstrated (Angel et al., 2013; Semenov et al., 2020; Angel et al., 2013; Semenov et al., 2020). Earthworm casts were obtained from mesocosms where *L. terrestris* was feed in an agricultural soil with straw material. Since food sources may strongly affect the consumption rate and cast production as well as microbial successions (Flegel et al., 1998; Whalen and Parmelee, 1999; Scullion et al., 2003), we compared straw of sainfoin and winter wheat, i.e. substrates with contrasting C/N ratios. Freshly produced casts were compared to the surrounding soil, and the ageing process of the casts was followed over a period of 168 d, either kept under constant moisture conditions or under dry-wet cycles at 28-d intervals. Previous studies with a *Lumbricus*-relative indicated that such dry-wet cycles inhibit microbial survival in casts and thereby hinder decomposition (Law and Lai, 2021).

We tested the following hypotheses: (i) The earthworm gut transit increases the abundances of bacteria, archaea and fungi due to a facilitated microbial access to energy rich organic substrates released from the food sources and intestinal mucus addition. (ii) The composition of the prokaryotic soil microbiome changes during the gut transit by favoring fast-growing (copiotrophic) community members and diets differing in their C/N-ratio will promote the growth of different taxa. (iii) Compositional changes of the prokaryotic microbiome are pronounced at both the structural level, as indicated by DNA, and at the level of activity, as indicated by rRNA-based analyses. (iv) Dry-wet cycles select for a different succession of prokaryotic taxa and slow down the ageing process, thus enhancing the legacy effects of the earthworm gut transit on the soil microbiome.

2. Materials and methods

2.1. Experimental design

2.1.1. Soil, earthworms and mesocosms

The bulk soil used in this study was a silt loam soil (12 % clay, 85 % silt, 3 % sand) derived from loess (Luvisol) which was collected from the surface (ca. 10 cm) of a cropland field site (9°56′ E 52°00′N, 196 m a.s.l.) near Hildesheim, Northern Germany, on July 12, 2021. The soil had a pH of 6.5, a total organic carbon content of 14.4 mg g^{-1} soil dry weight and total nitrogen of 1.5 mg g^{-1}. The soil was air-dried at room temperature overnight, then sieved through 2-mm mesh size and subsequently moistened by spraying to a water content of 22 % (w/w), corresponding to a water holding capacity (WHC) of 60 %. Adult *L. terrestris* specimen were purchased from a commercial supplier (Angelsport A&M, Braunschweig, Germany). For adaptation of the earthworms to the different diets, they were transferred to mesocosms containing the soil, covered either with straw of the legume sainfoin (*Onobrychis viciifolia* Scop., Fabaceae) or winter wheat (*Triticum aestivum* L., Graminaceae), respectively. The added earthworms had an average weight of 4.98 ± 1.05 g (n = 20). Five replicate polyethylene containers (size 20 cm × 9 cm x 10 cm) served as mesocosms, each was filled with 1.2 kg of soil, and to each of them, two earthworm specimens were added. Eight grams of dried straw of winter wheat (C content 45.3 % (wt/wt); N content 0.45 %) or sainfoin (C 46.6 %; N 2.1 %) were placed onto the soil surface in each container (Fig. S1). All containers were then covered by lids to prevent escaping of the earthworms and incubated under darkness in a climate chamber at 16 °C (±1 °C). The incubation period lasted 4 weeks, after which the weight of the earthworms was determined again.

2.1.2. Microcosms setup

For a total period of 2 weeks, each day, freshly produced casts deposited by the earthworms on the soil surface were collected using sterilized tweezers. In parallel, as a control, bulk soil from the vicinity of the casts but apparently not affected by earthworm activities, was also collected. A total of ca. 2 g of soil or cast (wet weight) were then immediately transferred to a small sterile polypropylene petri dishes (35 mm in diameter) which served as microcosms for the ageing experiments. For each sampling point and treatment, four replicate samples were analyzed. The following treatments were tested: (i) casts as compared to surrounding bulk soil; (ii) straw of sainfoin (SA) as compared to winter wheat (WW); (iii) constant moisture maintaining the initial water content of the samples as they were collected from the mesocosms (T1) as compared to dry wet cycles at 4-week intervals (T2). Microcosms were incubated at 21 °C under the respective moisture regime, and samples were collected by sacrificing the respective microcosms after 0, 3, 7, 14, 28, 84, and 168 d, respectively.

2.2. Nucleic acid extraction

DNA and RNA were co-extracted from 250 mg soil samples (wet weight) using ZymoBIOMICS DNA/RNA miniprep kit including bead beating steps following the protocol recommended by the manufacturer (ZymoResearch, Freiburg, Germany). DNA was quantified with Pico-Green (Quant-iT™ dsDNA Assay-Kits, Molecular Probes, Eugene, Oregon) and stored at −80 °C for molecular analysis. The quality and concentration of RNA was assessed by NanoDrop. A total of 100–1,000 ng RNA was reversely transcribed to single strand cDNA using the Maxima H Minus First Strand cDNA Kit (Thermo Fisher Scientific, Roskilde, Denmark) with random hexamer primers. Synthesized single stranded cDNA was stored at −80 °C for sequencing. The DNA and cDNA were diluted to ca. 10 ng μl^{-1} for the subsequent operations.

2.3. Quantitative PCR

Quantitative real time PCR (qPCR) was used to estimate the abundances of bacteria, archaea and fungi using a Bio-Rad CFX384 Real time PCR cycler with C1000 Touch (Biorad, Feldkirchen, Germany). Standard curves were generated in 3 replicates using 1:10 serial dilutions of quantified PCR products dissolved in DNase/RNase-Free distilled water (Thermo Fisher Scientific). Extracted DNA of type culture strains carrying the target genes were used as templates for PCR standard curves. The bacterial, archaeal and fungal culture strains: *Bacillus subtilis*, *Methanobacterium oryzae* (DSM 11106) and *Fusarium culmorum* (DSM 62191) were obtained from the DSMZ, Braunschweig, Germany. Corresponding 16S rRNA genes of bacterial and archaeal community were quantified via primer pairs BAC338F/BAC805R and ARC787F/ARC1059R, respectively (Yu et al., 2005). Fungal abundance was quantified by targeting a fragment of ITS1 region via primer pair NSII/58A2R (Martin and Rygiewicz, 2005). All qPCR reaction mixtures (10 μl) were composed of 5 μl SYBR Green PCR Master Mix (2×) (Maxima SYBR Green qPCR, Master Mix 2× no ROX, Thermo Fisher Scientific, Erlangen, Germany), 0.5 μl of each primer (10 μM), 3 μl of DNase/RNase-Free distilled water and 1 μl diluted-DNA (ca. 10 ng μl^{-1}) template. The PCR started with denaturation at 95 °C for 10 min, followed by 40 cycles denaturation at 95 °C for 15 s, annealing for 30 s at 55 °C for bacterial and archaeal abundances and 52 °C for fungi, extension for 30 s at 72 °C and at 79 °C for 15 s. A final extension process for 5 min, followed by the process of melting curve detection by heating the samples to 95 °C for 1 min, cooling at 55 °C for 1 min and increased to 95 by 0.5 °C per step with continuous fluorescens measurement. Amplification efficiencies were ca. 96 %–100 % for bacteria and archaea quantification, ca.86 % for fungi, and the correlation coefficients were above 0.99.

2.4. PCR amplification for amplicon sequencing and bioinformatic analyses

For PCR amplicon sequencing, the V4 region of the 16S rRNA/rDNA genes of bacteria and archaea was amplified by a one-step PCR using barcode-encoded-primer 515F/806R. Twenty-five μl reaction mixtures contained 5 μl 5× Q5 reaction Buffer, 0.5 μl of 10 mM dNTPs (200 μM/0.2 mM), 1.25 μl of 10 μM each primer tagged with barcodes (~bp), 0.25 μl of Q5 DNA polymerase (0.02 U/μl), 2 μl of DNA-template and 14.75 μl of nuclease free water; while additional 5 μl 5 × Q5 GC enhancer was added into the reaction mixtures for amplifying cDNA template and adjusted with nuclease free water to 9.75 μl. PCR products were normalized, purified and pooled together afterwards. Pooled purified PCR amplicons were sequenced on the Illumina MiSeq platform by LGC Genomic (Berlin, Germany). All DNA and cDNA samples were sequenced in the same run, each with 15 Million paired-reads. Due to the blind ligation of Illumina adaptor and amplicon fragments, the raw sequence reads were reoriented into respective forward and reverse reads files (github.com/DamienFinn/MiSeq_read_reorientation) before being analyzed on QIIME2 platform (Bolyen et al., 2019).

Reoriented forward and reverse reads were subjected to quality filtering, removal of primers, merged and denoised with DADA2 (Callahan et al., 2016) using Qiime2 pipeline truncated at the position base 30 and 280 of the ca. 300 bp amplicon. The Silva 138 database (Quast et al., 2013) was used to assign the higher resolution of sequences into amplicon sequence variants (ASV) for taxa analysis. The taxa identified as eukaryote-associated (mitochondria and chloroplasts) were removed from the dataset. The raw DNA sequences have been deposited at the NCBI database (Project Accession number: PRJNA957594).

2.5. Co-occurrence network analyses and identification of keystone taxa

Network analyses were conducted to assess the temporal dynamic co-occurrence patterns of ASV using the "WGCNA" R package (Langfelder and Horvath, 2008). ASV below 0.02 % relative abundance were filtered out to avoid spurious correlations. Spearman's rank values were used for calculating the association among microbial ASV from all time-point. False discovery rate correction was used for correcting P-value from multiple comparison. Correlation coefficients greater than 0.7 with a corresponding P-value less than 0.05 were considered as statistically robust and were included to construct the networks. The networks were visualized via the "igraph" package with the Fruchterman-Reingold layout, and their topological properties were also calculated with this package. Keystone taxa are defined as nodes with high degrees but low betweenness centralities. In our study, ASV with high degrees ranked at top 15 and normalized betweenness centrality lower than 0.025 were defined as keystone taxa.

2.6. Statistical analysis

All statistical analyzes and visualizations were performed in R version 4.1.2 (Team, 2021). Alpha diversity (Shannon-index, richness and evenness) was calculated by rarefied sequences with equal depth, the calculation and rarefaction were conducted with "vegan" package R version 4.1.2 (Oksanen et al., 2020). Statistically significance in physicochemical soil parameters (C and N) and microbial abundances were tested by Tukey's HSD test in R. Fisher's Least Significant Difference (LSD) post hoc test was applied to test the significance of alpha diversity in fresh components (soil vs cast) from different feeding diets via the "agricolae" package (de Mendiburu, 2021). Principal coordinate analysis (PCoA) plots based on Bray-Curtis dissimilarity were calculated via "vegan" package at ASV-level. Permutational multivariate analysis of variance analysis (PERMANOVA) was performed to assess treatment differences by following 999 permutations (adonis function). Center log ratio (CLR) transformed relative abundances of taxa at Phylum-level that calculated in "funrar" package were visualized as heatmaps via the "gplots" package (Warnes et al., 2009). T-test was used to test the effect of earthworm gut passage and diets on abundances of taxa. Univariate general linear models (glms) were applied to test for ageing on the detection of each Phylum. Ageing effects interactinged with food sources and moisture on microbial dynamic changes at Phylum level were examined via linear mixed-effect model in which ageing time was set as a fixed factor.

3. Results

3.1. Effect of different diets on earthworm weight and physicochemical properties of their casts

At the end of mesocosm incubation of 4 weeks for collecting fresh casts, the earthworms had lost with WW as a diet 11.2 ± 8.9 % weight of their initial biomass, while with SA as the diet there was no comparable weight loss with 0.89 ± 1.9 %. The amount of cast shed by the earthworms per day per mescosm was 2.6 g ± 0.6 g with WW and 3.0 g ± 0.4 g with SA, respectively.

Irrespective of the diet, the pH of freshly produced casts was 6.0, and thus lower than the surrounding bulk soil with pH 6.4. The C and N contents of SA casts were higher with 25.8 mg C g^{-1} dry weight and 2.9 mg N g^{-1} as compared to WW casts with 19.0 mg C g^{-1} and 1.9 mg N g^{-1} (Fig. S2). The similar C/N ratios in casts with 8.9 (SA) and 10.0 (WW) contrasts the differences which were associated with the original straw material with SA (22.2) and WW (100.7), respectively. The C contents of SA cast was 1.8-fold and of WW cast 1.3-fold higher than in the surrounding bulk soil. During cast ageing over a period of 168 d, their C contents tended to decline irrespective of the straw and moisture conditions (Fig. S2). The N contents of SA declined but for WW and bulk soil it tended to slightly increase. Moisture conditions had no tangible effects on this trend.

3.2. *Effect of gut passage and ageing on microbial abundances*

Gene copy numbers determined by qPCR from total DNA extracted from freshly shed casts indicated a higher abundance of bacteria and fungi, but lower abundance of archaea as compared to the surrounding bulk soil (Fig. 1). These effects were significant except for fungi in casts from mesocosms with WW (Table S1). While the earthworm diet had a significant effect on bacterial gene copy numbers, indicating higher abundance with SA as compared to WW, it had no effect on the abundances of archaea or fungi (Table S1).

Differences of microbial abundance between cast and bulk soil became less tangible during the cast ageing process, and they were affected by the moisture conditions (Table S2). For bacteria, the last significant effect between cast and bulk soil under constant moisture (T1) was detected after 7 d with SA and 14 d with WW, but with dry-wet cycles (T2) differences persisted until 28 d. This effect was even more pronounced for archaea, where differences under constant moisture persisted for 14 d, while with dry wet cycles, differences were maintained throughout the experiment until the end of observation after 168 d. Similarly, with dry-wet cycles differences in fungal abundance between casts and soil were maintained throughout the 168 d, but this effect was only seen with SA as a food source. In contrast, under constant

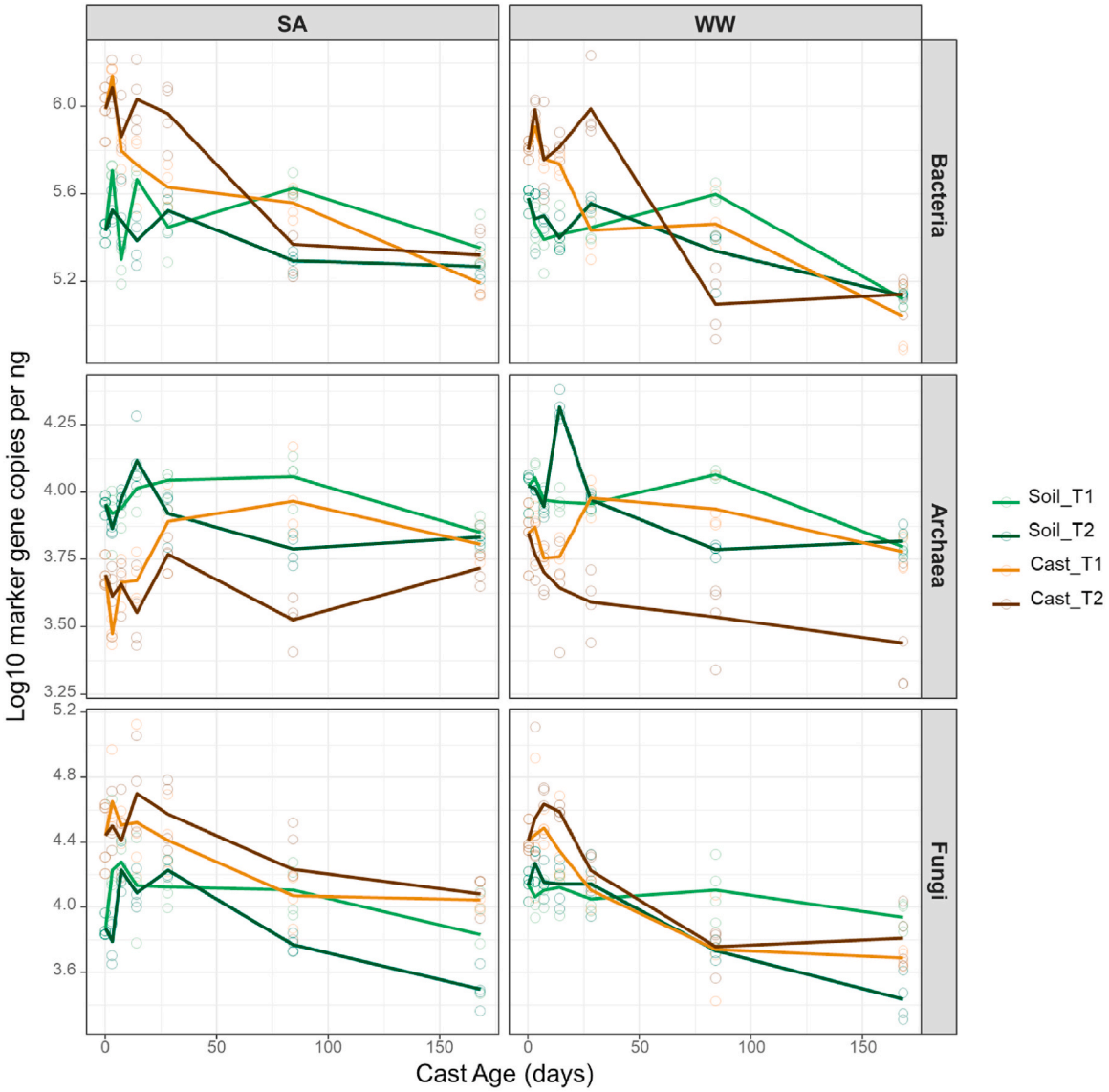

Fig. 1. Copy numbers of marker genes to quantify the abundance of microbial communities in soils and earthworm casts along the ageing time, as assessed by qPCR from directly extracted soil DNA, targeting the 16S rRNA genes of bacteria and archaea and the fungal ITS1 sequences, respectively. SA: sainfoin; WW: winter wheat. T1: constant moisture; T2: dry-wet cycles at 4-week intervals. Results of their statistical analyses are shown in more detail in the Supplemental Material (Tables S1 and S2).

soil moisture, differences of fungal abundance between cast and bulk soil, irrespective of the diet, vanished after 7 d. Thus, overall, dry-wet cycles apparently stabilized differences triggered by the gut passage much stronger than under constant soil moisture conditions.

3.3. Differences in the composition of the prokaryotic microbiome from bulk soil and freshly produced casts – comparison of DNA and RNA levels

Comparable libraries of 16S rRNA gene and 16S rRNA cDNA amplicons were obtained from the bulk soil and cast samples of this study (Supplemental Material R.S1, Table S3). Considering all ASV, at the DNA level, Shannon diversity was not significantly modified by the gut transit, while at the RNA-level a lower diversity was detected in WW casts but not SA casts, as compared to bulk soil (Table S4). For both, DNA and RNA level, evenness was slightly and significantly lower in casts as compared to bulk soil, but richness values were not different. The phylogenetic assignment of ASV to the phylum level from the bulk soil samples are indicated in Table 1 and described in more detail in Supplemental Material R.S2, Tables S5 and S6).

At the DNA level, freshly produced casts showed a similar relative abundance of Proteobacteria and Actinobacteriota as in the surrounding bulk soil, but a strong increase in Bacteroidota and a decrease of Acidobacteriota and Crenarchaeota as well of the less abundant phyla Chloroflexi and Gemmatimonadota (Table 1). However, for Proteobacteria, differences between cast and bulk soil became evident at the class level: In casts, Gammaproteobacteria had a higher relative abundance than Alphaprotobacteria, while in bulk soil it was the opposite (Table S7).

In contrast to DNA, the RNA level also indicated an increase for the phylum Proteobacteria, for which Gammaproteobacteria were exclusively responsible (Table S7 a.). All four orders analyzed here, including Pseudomonadales and Enterobacteriales contributed to this stimulated response (Table S7 b.). With RNA, Actinobacteriota showed lower relative abundance in casts than in bulk soil. Similarly, and in line with the DNA data, a lower relative abundance in casts as compared to bulk soil was also detected with Acidobacteriota and Crenarchaeota. With RNA, but not with DNA, a higher relative abundance of Verrucomicrobiota was detected in casts. For Bacteroidota both DNA and RNA level indicated that the increase of their relative abundance in casts was mainly caused by Flavobacteriales (Table S8).

For the earthworm diet, no tangible effects were detected at the DNA-level when comparing the relative abundance of the 20 most dominant prokaryotic phyla in freshly produced casts from mesocosm with SA and WW (Table 1). In contrast, with rRNA, the relative abundance of Proteobacteria (affiliated with Gamma- but not Alphaproteobacteria; Table S7) was significantly higher in SA as compared to WW casts. Other phyla, including Actinobacteriota, Bacteriodota or Verrucomicrobiota also showed strong differences in mean values between both diets, but these were not significant.

Table 1
T-test of relative abundances (mean values) of the most dominant 20 prokaryotic phyla in freshly produced earthworm casts and surrounding bulk soil. Values in bold represent significant differences in relative abundances between bulk soil and cast ($p < 0.05$). Values in italics represent the significant effects of diets ($p < 0.05$). SA: sainfoin; WW: winter wheat. Values with * indicate significances in relative abundances between DNA and RNAlevel. The phylum indicated with (D) was only detected with DNA and the one with (R) only with RNA-based detection.

Phylum	DNA			RNA		
	Bulk soil	SA-cast	WW-cast	Bulk soil	SA-cast	WW-cast
Proteobacteria	33.3*	39.2	37.0	**23.7**	***44.3***	***34.2***
Actinobacteriota	**21.7***	**16.9**	**20.0**	**27.0**	**11.7**	**14.1**
Bacteroidota	**3.7***	**25.2**	**21.3**	**1.4**	**15.9**	**17.2**
Acidobacteriota	**12.4***	**2.4**	**3.5**	**5.2**	**1.9**	**2.4**
Crenarchaeota	**8.6***	**2.3**	**3.5**	**12.1**	**5.7**	**5.1**
Firmicutes	4.0*	3.9	4.4	4.6	4.2	5.0
Verrucomicrobiota	4.9*	3.9	4.9	**2.6**	**6.9**	**10.4**
Chloroflexi	**4.2**	**1.1**	**1.5**	**3.2**	**1.3**	**2.0**
Myxococcota	1.8*	1.2	1.0	**8.8**	**1.9**	**5.3**
Planctomycetota	1.3*	0.6	0.6	**5.3**	**1.6**	**2.3**
Gemmatimonadota	**1.2***	**0.2**	**0.2**	**0.4**	**0.1**	**0.1**
Nitrospirota	**1.2**	**0.3**	**0.4**	**0.8**	**0.2**	**0.4**
Methylomirabilota	**0.7***	**0.1**	**0.1**	**1.6**	**0.2**	**0.3**
Desulfobacterota	0.2	0.0	0.0	**0.3**	**0.0**	**0.0**
Entotheonellaeota	0.2	0.0	0.1	**2.4**	**0.3**	**0.5**
Bdellovibrionota	0.1	0.1	0.0	0.4	0.3	0.2
Cyanobacteria	0.1	0.1	0.0	0.0	0.0	0.0
Latescibacterota	0.1	0.0	0.0	**0.1**	**0.0**	**0.0**
Patescibacteria (D)	0.0	0.1	0.0	0.0	0.0	0.0
Armatimonadota (R)	0.0	0.0	0.0	0.1	0.0	0.0
Unassigned	0.0	2.4	1.1	0.0	3.3	0.5

3.4. Detection of microbial growth and death during the gut passage

By combining qPCR of 16S rRNA gene copies and relative abundance data at DNA level from surrounding soil and freshly shed casts, we estimated microbial growth rates during the gut transit assuming that an average gut passage time in *Lumbricus terrestris* would be 12 h (Taylor and Taylor, 2014) (Table S9 a.-c.). Highest growth rates were seen for Bacteroidota, and therein, for the orders Flavobacteriales and Sphingobacteriales. The highest rate of 0.153 h^{-1} for Flavobacteriales would correspond to a doubling time of 2.0 h. Highest death rates were detected for Crenarchaeota with −0.072 h^{-1}, corresponding to a time of ca. 4.2 h for a 50% reduction.

Differences between growth rates caused by the two alternative diets were apparent, e.g. with SA promoting growth of Bacteriodota, Proteobacteria, Myxococcota and Planctomycetota more strongly than with WW. This was also seen at the order level, e.g. for Flavobacteriales (Table S9b), or within Bacilli for Bacillales and Paenibacillales (Table S9c). On the other hand, for five of six phyla, including Gemmatimonadota and Methylomirobilota, death rates were higher with WW as compared to SA.

3.5. Effect of cast ageing on the composition of their microbiomes

The implications of the cast ageing process on the composition of the prokaryotic microbiome, as affected by diet and soil moisture conditions, were studied at both, DNA and the RNA level. Here, we considered the ten most dominant phyla.

At the DNA level, as compared to the surrounding bulk soil controls, the gut transformations affected the dynamics in the relative abundance of six of ten phyla under continuous moisture (T1), while it affected eight phyla with dry-wet cycles (T2) (Fig. 2 a. and c.). During the ageing process, different dynamics between SA or WW derived casts were revealed for two phyla under T1, and for four phyla under T2 conditions (Fig. 2 b. and d.). More specifically, the dynamics of Actinobacteriota was only different between SA and WW under T1 conditions, while Crenarchaeota, Firmicutes and Myxococcota had a significantly different dynamic only under T2. Irrespective of the moisture regime, Acidobacteriota were affected differently by SA and WW as a food source.

In comparison to the DNA level, with RNA-based analyses only five phyla were affected in the dynamics of their relative abundance during cast ageing, under T1 and six phyla under T2 conditions, with an overlap of four phyla affected by both (Fig. 3 a. and c.). Acidobacteriota were only affected at constant moisture (T1) while Myxococcota and Proteobacteria were only significantly affected with dry-wet cycles (T2).

Casts obtained from earthworms fed with SA as compared to WW showed differences for a total of four different phyla. Regardless of the moisture conditions Acidobacteriota and Firmicutes differed during ageing between SA and WW (Fig. 3 b. and d.). In contrast, Actinobacteriota showed differences between SA and WW only under constant

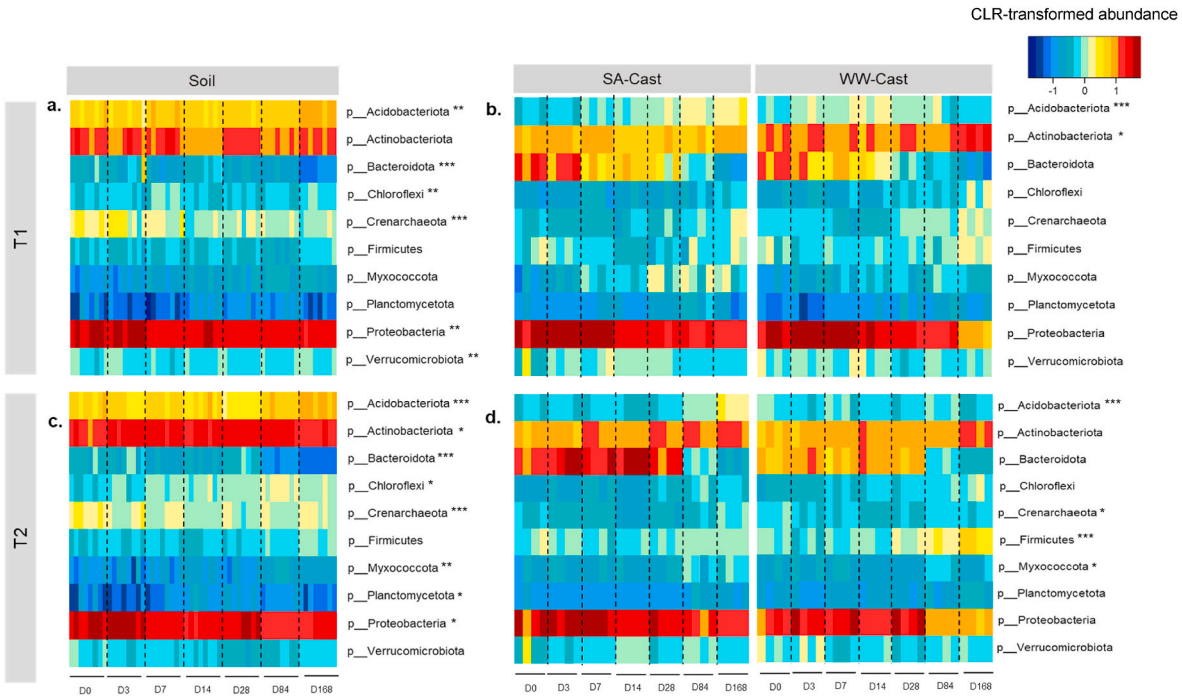

Fig. 2. Heatmaps of center log ratio (CLR) transformed relative abundances of the ten most abundant prokaryotic phyla in soil and casts across ageing time at **DNA level**. Mixed-effect linear models (glms) were used for testing the effect of ageing co-influenced by gut transformation (changes of phyla differed between soil and cast indicated by asterisks labelled in **a.** & **c.**) and diet (changes of phyla differed between SA- and WW-derived casts, indicated by asterisks labelled in **b.** & **d.**). Levels of significance indicated: $P < 0.001$: ***; $P < 0.01$: ** and $P < 0.05$: *. SA: sainfoin; WW: winter wheat; T1: constant moisture; T2: dry-wet cycles at 4-week intervals; D0-D168: sampling day. Results of statistical analyses (glms) of ageing effects on each Phylum are shown in more details in the Supplemental Material (Table S10).

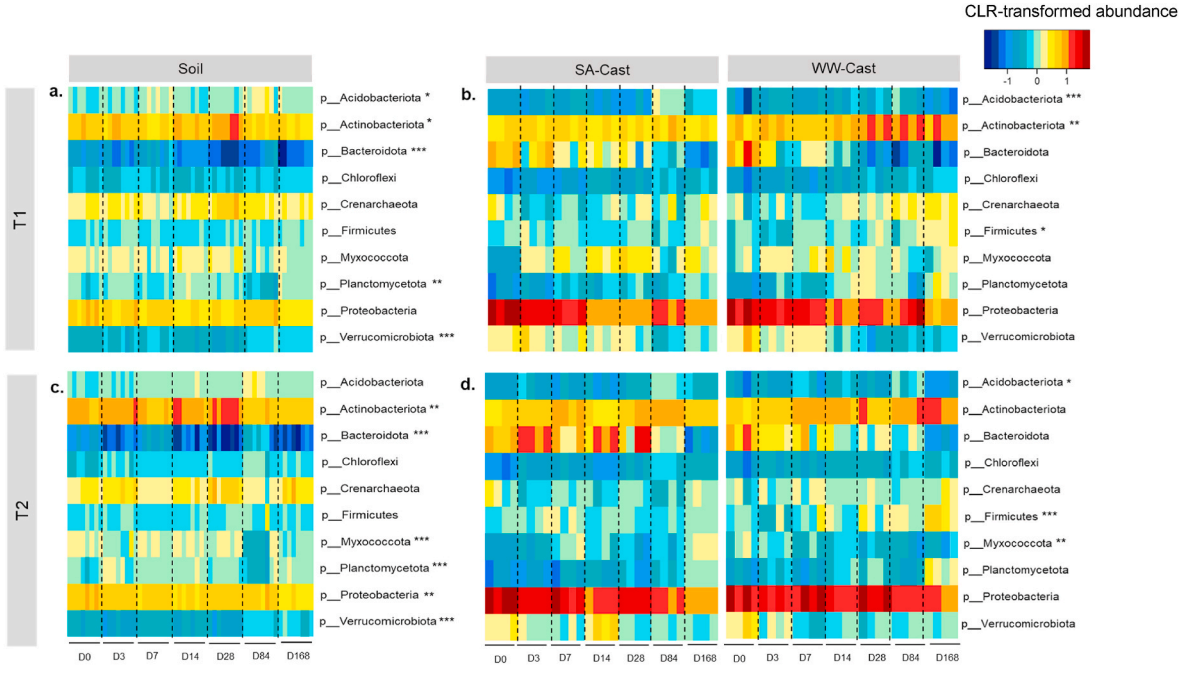

Fig. 3. Heatmaps of center log ratio (CLR) transformed relative abundances of the ten most abundant prokaryotic phyla in soil and casts across ageing time at **RNA level**. Mixed-effect linear models (glms) were used for testing the effect of ageing co-influenced by gut transformation (changes of phyla differed between soil and cast indicated by asterisks labelled in **a.** & **c.**) and diet (changes of phyla differed between SA- and WW-derived casts, indicated by asterisks labelled in **b.** & **d.**). Levels of significance indicated: $P < 0.001$: ***; $P < 0.01$: ** and $P < 0.05$: *. SA: sainfoin; WW: winter wheat; T1: constant moisture; T2: dry-wet cycles at 4-week intervals; D0-D168: sampling day. Results of statistical analyses (glms) of ageing effects on each Phylum are shown in more details in the Supplemental Material (Table S10).

moisture conditions, and Myxococcota only with dry-wet cycles, respectively.

Mixed linear models based on center log ratio (CLR) transformed relative abundance data confirmed that the majority of phyla significantly change during the ageing process at both DNA and RNA level (Table S10). Compositional changes were also seen in the surrounding bulk soil. Responses of Verrucomicrobiota were only detected at the RNA level.

Considering the compositional data obtained from the DNA and RNA level based on ASV, PCoA revealed distinct communities for both, separated along PCo2, with more variation between samples at the RNA level (Fig. 4). Along PCo1 there was for both DNA and RNA compositional data, a strong separation between freshly shed cast samples and the surrounding bulk soil. As the ageing process proceeded, the samples from casts became more similar to those from bulks soil. PERMANOVA accordingly indicated for both, DNA and RNA level, a significant effect of ageing on the community composition, and, beyond that also for moisture conditions and food type, respectively (Table 2). These effects are also tangible with faceted PCoA, comparing each individual time point (Fig. S4).

3.6. Network analyses

Co-occurrence networks were constructed for analyzing the compositional changes during the 168-d incubation period separately for bulk soil, SA casts and WW casts, and for both moisture conditions, respectively. Networks were constructed for ASV obtained from both DNA (Fig. 5) and RNA (cDNA) (Fig. 6). No substantial networks were detected from bulk soil samples, because of only minor compositional variation, thus indicating fairly stable communities over time. In contrast, the networks of cast microbiomes were complex and their topological features were affected by both diets (SA, WW) and moisture conditions (T1, T2). Generally, and irrespective of DNA or RNA, networks of WW-cast had a higher complexity (more edges and nodes; more positive and negative links) compared to SA-cast-network (Table 3). The complexity of the networks was also enhanced under dry-rewet cycles as compared to continuous moisture (except for SA-cast at RNA level aged under T1). The detailed characteristics of the resulting networks are available in the Supplemental data file "Table S11").

The prokaryotic microbiomes at the DNA or RNA level showed that the major keystone taxa in the temporal microbial networks of SA-cast

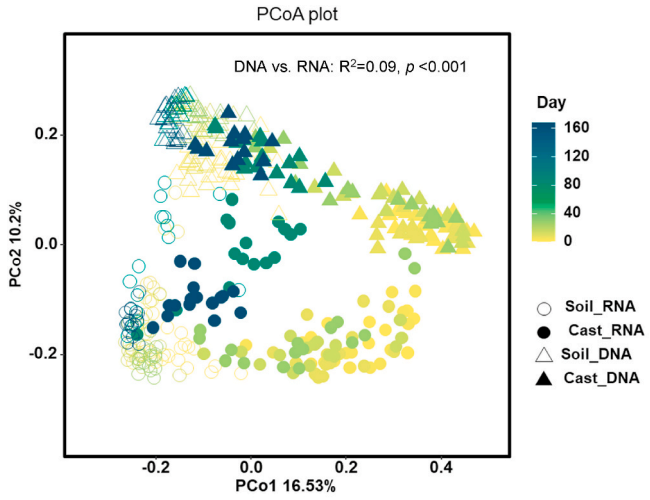

Fig. 4. Principal coordinate analysis (PCoA) plot based on Bray-Curtis dissimilarity showing compositional changes of the prokaryotic communities at the DNA and RNA level during the ageing process of casts. PERMANOVA results testing for the effect of different target groups (DNA vs. RNA) on prokaryotic communities are inserted into the figure.

Table 2
Influence of effects of compartments (soil vs. cast), ageing, food type and moisture conditions on the beta-diversity of cast prokaryotic communities at both DNA and RNA level, as analyzed by PERMANOVA.

	DNA		RNA	
	R^2	P	R^2	P
Soil vs Cast	0.141	0.001	0.127	0.001
Ageing	0.05	0.001	0.037	0.001
Moisture	0.034	0.001	0.026	0.001
Diet	0.015	0.001	0.016	0.001

were affiliated with Bacteroidota (class Bacteroidia) and Proteobacteria (class Gammaproteobacteria). Under dry-rewet condition, one member of Myxococcota (genus *BIrii41*) and one Actinobacteriota (genus *Conexibacter*) were identified as keystone ASV at RNA level while for the DNA-level, one member affiliated with Nitrospirota (genus *Nitrospira*) and one affiliated with Acidobacteriota (family Vicinamibacteraceae) were detected. In contrast, the keystone members in the WW-derived cast belonged to more diverse phyla, including Verrucomicrobiota, Gemmatimonadota, Firmicutes or Actinobacteriota, which were detectable under both moisture conditions during the ageing period.

4. Discussion

The gut passage of soil amended with straw resulted in freshly shed casts which were characterized by higher C and N content as compared to bulk soil. This increase can be attributed to the degradation of the straw diet and the production of mucus by the earthworms (Drake and Horn, 2007; Blouin et al., 2013). The comparison of prokaryotic community abundance and composition indicated that strong modifications of the ingested soil community occurred within a relatively short period of time, i.e. on average ca. 12 h, as estimated *for L. terrestris* in another study (Taylor and Taylor, 2014). Irrespective of the diet, whether it was SA with a low C/N ratio or WW with high C/N, major beneficiaries were bacteria of the phylum Bacteroidota, and therein especially Flavobacteriales. Flavobacteria were also detected as dominant gut bacteria of *L. terrestris* in other studies (Nechitaylo et al., 2010; Buivydaite et al., 2023), suggesting that they are enriched from different soils and substrates. The estimated growth rates of Flavobacteriales, determined by us using a combination of qPCR and 16S rRNA gene amplicon sequencing indicated a generation time of only 2.0 h, thus allowing the population of the respective Bacteroidota to increase more than 16-fold within 12 h. Such doubling times of soil bacteria are indicative of a copiotrophic life-style (Fierer et al., 2007; Stone et al., 2023) and strongly contrast suspected generation times in bulk soil, the latter estimated to be between 6 and more than 1,000 days (Harris and Paul, 1994; Caro et al., 2023).

In addition to Bacteroidota, Proteobacteria and Firmicutes which encompass many known copiotrophs, were also able to grow during the gut passage, even though with lower generation times, with estimated maxima of 6.5 and 10.8 h, respectively. In common, many members of these three most responsive phyla have the capacity to grow under denitrifying conditions. In our study, Firmicutes were mainly represented by aerobic and potentially denitrifying Bacilli and Paenibacilli (Behrendt et al., 2010; Verbaendert et al., 2011; Behrendt et al., 2010; Verbaendert et al., 2011) and not by the strictly anaerobic Clostridia. This underlines the importance of this route of respiration by facultative anaerobes in earthworm guts, supporting previous findings (Horn et al., 2006a). Also, the loss of N from sainfoin as indicated by differences between the C/N ratio of the ingested substrate and the corresponding casts provides good evidence for ongoing denitrification.

On the other hand, there was a strong decline of Crenarchaeota, which were mainly represented by nitrifying *Nitrososphaera*, indicating that these aerobic taxa with the potential of nitrification and suspected

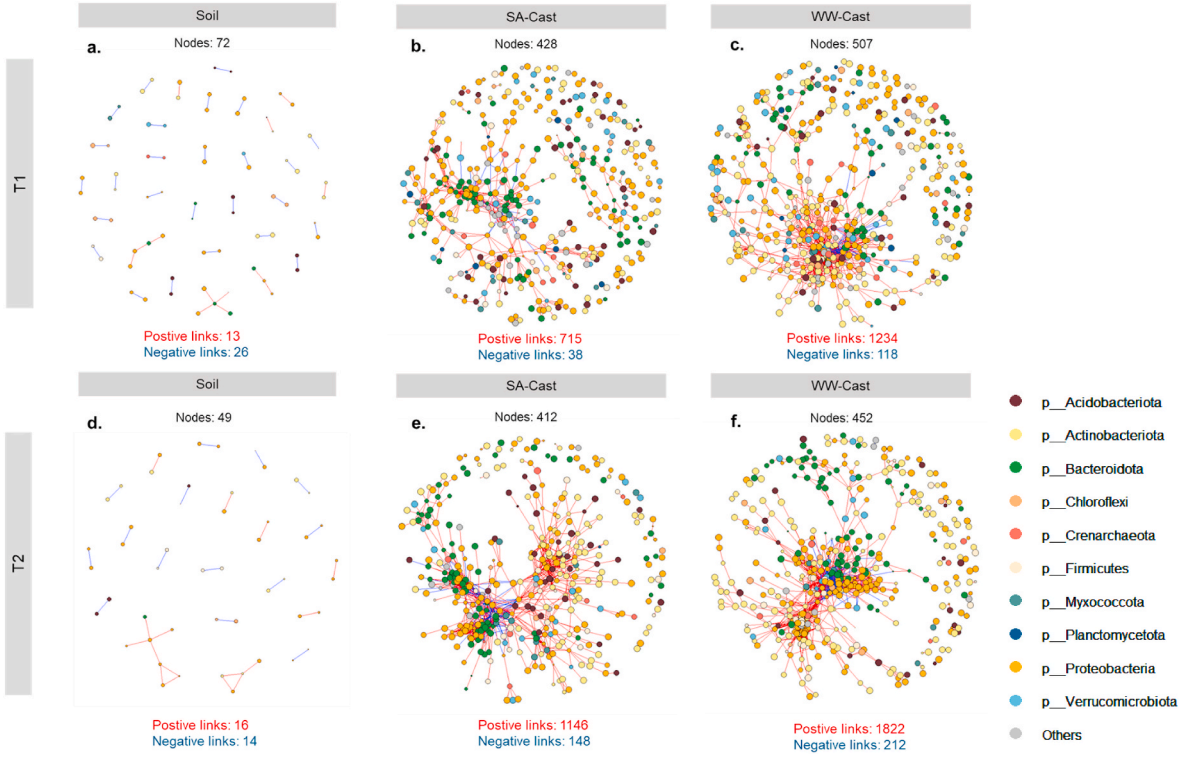

Fig. 5. (**a. – f.**). Co-occurrence networks of total (**DNA level**) prokaryotic communities at ASV level in earthworm casts and soils across treatments during the 168-d incubation. Only ASV with a Spearman's correlation coefficient ($r > -0.7$ or $r < 0.7$ and FDR adjusted $P < 0.05$) are considered. Each node represents an ASV. Color nodes indicate the contributing phyla and their sizes are proportional to their relative abundance. Red edges represent positive correlations and blue edges represent negative correlations. SA: sainfoin; WW: winter wheat; T1: constant moisture; T2: dry-wet cycles at 4-week intervals.

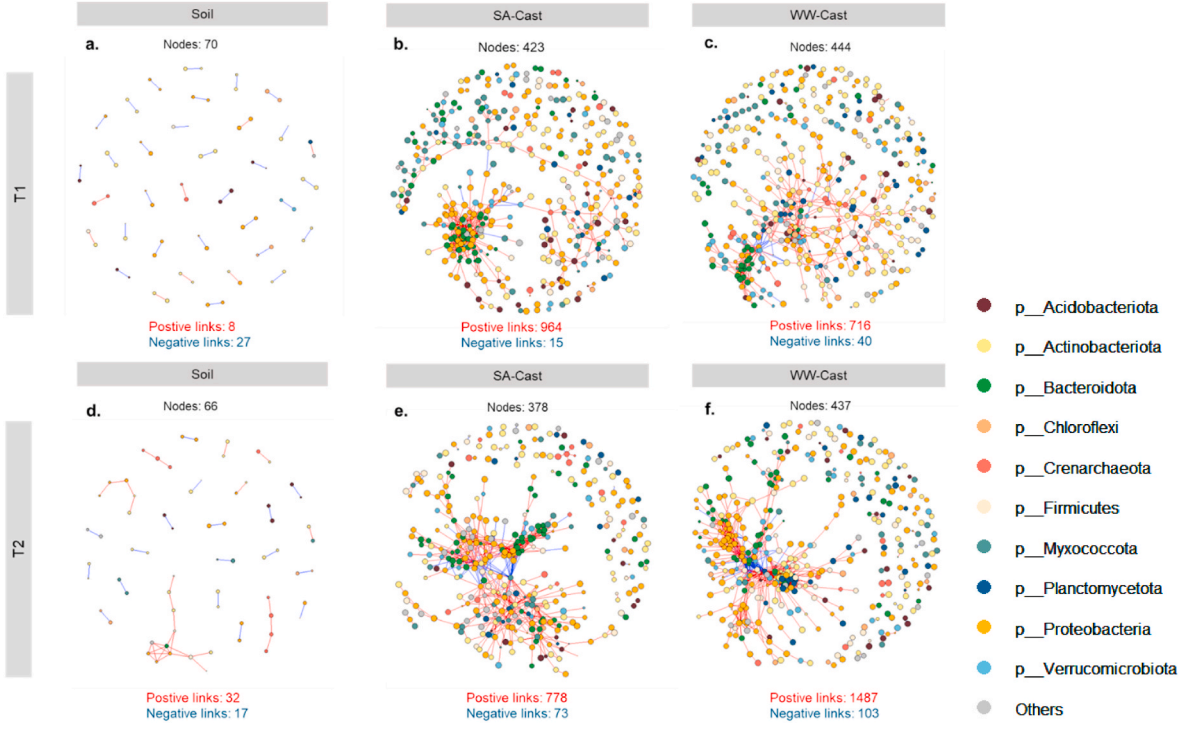

Fig. 6. (**a. – f.**). Co-occurrence networks of active (**RNA level**) prokaryotic communities at ASV level in earthworm casts and soils across treatments during the 168 d of incubation. Only ASV with a Spearman's correlation coefficient ($r > -0.7$ or $r < 0.7$ and FDR adjusted $P < 0.05$) are considered. Each node represents an ASV. Color nodes indicate the contributing phyla and their sizes are proportional to their relative abundance. Red edges represent positive correlations and blue edges represent negative correlations. SA: sainfoin; WW: winter wheat; T1: constant moisture; T2: dry-wet cycles at 4-week intervals.

Table 3
Summary of network topological properties of all networks at the DNA (**a.**) and RNA level (**b.**). SA: sainfoin; WW: winter wheat; T1: constant moisture; T2: dry-wet cycles at 4-week intervals.

a.						
DNA	T1			T2		
	Bulk soil	SA-Cast	WW-Cast	Bulk soil	SA-Cast	WW-Cast
Positive edges	13	715	1234	16	1146	1822
Negative edges	26	38	118	14	148	212
No. of nodes	72	428	507	49	412	452
No. of edges	39	753	1352	30	1294	2034
Clustering coefficient	0	0.41	0.39	0.38	0.43	0.58
Average degree	1.08	3.52	5.33	1.22	6.28	9
Average path length	1.22	6.18	6.24	1.48	4.81	5.28
Ratio of positive to negative edges	0.50	18.82	10.46	1.14	7.74	8.59
Proportion of positive edges %	33.33	94.95	91.27	53.33	88.56	89.58
Proportion of negative edges %	66.67	5.05	8.73	46.67	11.44	10.42
b.						
RNA	T1			T2		
	Bulk soil	SA-Cast	WW-Cast	Bulk soil	SA-Cast	WW-Cast
Positive edges	8	964	716	32	778	1487
Negative edges	27	15	40	17	73	103
No. of nodes	70	423	444	66	378	437
No. of edges	35	979	756	49	851	1590
Clustering coefficient	NA	0.48	0.43	0.55	0.43	0.61
Average degree	1.00	4.63	3.41	1.48	4.50	7.28
Average path length	1.00	10.13	6.62	2.12	4.70	4.60
Ratio of positive to negative edges	0.30	64.27	17.90	1.88	10.66	14.44
Proportion of positive edges %	22.86	98.47	94.71	65.31	91.42	93.52
Proportion of negative edges %	77.14	1.53	5.29	34.69	8.58	6.48

slow growth were not competitive. *Nitrososphaera* sp. can be the most dominant nitrifying taxa in agricultural soil (Lu et al., 2020), and apparently, they encounter adverse conditions inside the earthworm gut. In fact, their decline at the DNA level indicates that many of their cells apparently died (Blazewicz et al., 2014) and may have been digested during the gut passage. For serving as a significant nutrient source, however, to quantitative abundance and thus, the suspected biomass, was too small in comparison to the C, N and energy provided by the SA and WW straws.

In contrast to the decline of archaea, both bacterial and fungal populations increased in abundance, irrespective of whether SA or WW were supplied as the diet. Thus, also fungal growth was stimulated during the gut passage, and it can be suspected that both bacteria and fungi grew because they were capable of utilizing the supplied straw material being mainly composed of carbohydrates, i.e. cellulose, hemicelluloses and lignin as carbon and energy sources. Higher abundance of bacteria but not fungi with the N rich SA in contrast to N-depleted WW suggests that fungi were less efficient in presence of good N supply. This is in accordance with the experience that bacterial decomposers are better at lower and fungal at higher C/N ratios (Rousk and Baath, 2007).

Interestingly, the relative abundance of Actinobacteria declined during the gut passage, but quantitative estimates, on the other hand, clearly indicated their growth, even though slower with generation times of 9.7 and 14.2 h. Actinobacteriota are well-known for their lignolytic activity (Taylor et al., 2012) and it can be expected, due the complex molecular structure and its relatively high environmental persistence, that this fraction decomposes also more slowly in the earthworm gut as compared to the cellulose containing C fractions. The decline of the relative abundance of Actinobacteriota as result of slower growth compared to Bacteroidota or Proteobacteria, but certainly not death, demonstrates how misleading direct comparisons of relative abundances data can be interpreted (Gloor et al., 2017).

The two earthworm diets supplied in this study, i.e. SA and WW, did not only differ in their C/N ratio, but also in their palatability for the earthworms. With SA the earthworms did not lose body weight during the four weeks of feeding in the mesocosms while with WW it declined by 11.2%, which is still below the tolerable biomass loss of 20% in laboratory experiments (Fründ et al., 2010). Probably the earthworms could utilize this higher N supply as an important nutrient and were therefore healthier than under N-limitation with WW. The higher bacterial but not fungal or archaeal abundance in SA may also reflect a healthier gut microbiome for the earthworms, because conditions inside the earthworm gut are generally not favorable for fungal and archaeal growth and survival (Moody et al., 1996); Hoeffner et al., 2018). For prokaryotes, compositional differences between casts of SA and WW were only detected at the RNA level, where Proteobacteria increased with SA more than with WW, demonstrating that they became active during the gut passage and probably received more nutrients from SA than from WW. Gammaproteobacteria, including Pseudomonadales, but also Enterobacteriales, were the main responders to these better nutrient sources, while Alphaproteobacteria generally declined. While conditions of denitrification were very suitable for Pseudomonadales, Enterobacteriales may have contributed with fermentation. This would explain also the lower pH which was found in casts as compared to the surrounding bulk soil. Enterobacterial fermentation in the gut of *L. terrestris* has also been demonstrated in another study (Wüst et al., 2011). The positive effect of better dietary N supply was also tangible with generation times of Proteobacteria which were 6.5 h with SA and 10.8 h with WW. In contrast, no bacterial taxa (ASV) could be identified which grew faster with WW than with SA, confirming that under N-limited conditions a different, somewhat more oligotrophic microbiome was selected.

Differences between SA and WW were also detected for death rates, e.g. Crenarchaeota more strongly decreased with SA, probably as a result of being less competitive under the nutrient rich and oxygen depleted conditions. For other phyla, like Chloroflexi or Gemmatimonadota, death rates were higher with WW. Together these effects underlined that the quality of straw or its C/N ratio can have complex outcomes on the cast microbial communities. Compositional changes in cast microbiomes were also detected in other studies (Scullion et al., 2003), demonstrating that diets do not only modify the physicochemical properties of casts (Flegel et al., 1998; Vidal et al., 2019) but also their associated microbiomes. This has also recently been shown for the endogeic earthworm *Aporrectodea caliginosa* (Aira et al., 2022).

The ageing process of casts was followed in this study over a period of 168 d. During this time, bacterial and fungal abundances declined and archaeal abundances increased reaching the levels detected in the bulk soil controls. The strongly modified composition of the prokaryotic microbiome in fresh casts become more similar to the surrounding bulk soil, as indicated at the DNA and RNA level with multivariate statistical analyses. Generally, there was more dissimilarity between RNA- than DNA-based profiles, reflecting higher variation in activity as compared to structure during the ageing process. However, despite becoming more similar to bulk soil, even after 168 d, cast microbiomes were still distinguishable from those in bulk soil. Apparently, the gut passage had a tangible effect on microbiomes which lasted longer than a typical vegetation period, affecting both their structure (DNA) and activity (RNA). The long-term persistence of the physical cast structures (Marashi and Scullion, 2003; Vidal et al., 2019) apparently also provides protected microhabitats for specific soil microbiomes.

While heatmaps indicated that the diets, SA or WW showed different compositional dynamics, an even more pronounced effect was revealed by the contrasting moisture conditions. The water-limiting conditions

preserved the quantitative differences between cast and bulk soil, and PERMANOVA indicated that the compositional changes caused by dry-wet cycles had a stronger effect on the ageing process than the diets. These may be explained by that the repeated dry-wet cycles could directly change structural stability of the casts and reduce cast microbial survival during the drying periods, which subsequently led to the inhibitions of the rates of C and N mineralization in casts during ageing (Law and Lai, 2021). Thus, the water perturbation during the cast ageing process was more likely to be a primary factor affecting the microbial metabolic and compositional dynamic changes rather than the diets which primarily contributed to the variations of nutrient contents and compositions in fresh casts.

Network analyses were applied to compare the compositional changes during the ageing process and identify differences caused by diets and moisture conditions. Irrespective of the DNA or RNA level, networks from WW casts had a higher complexity than those from SA, suggesting that under N-limiting conditions more interactions between community members were supported as compared to better growth conditions with more N supply, as the latter may have favored competitive growth. There were both more positive and negative links, thus indicating that also a loss of one taxon may have specifically triggered the loss of others, while in less connected networks this loss would be more variable and random. While the keystone taxa of SA were representatives of the most dominant phyla (Bacteroidota and Proteobacteria), they were much more diverse with WW, including Verrucomicrobiota, Gemmatimonadota, Firmicutes or Actinobacteriota. This suggests that a more diverse community evolved under the N-limiting conditions. More complex networks under N limitations were also recently reported for an agricultural soil with a diversified cropping system (Liu et al., 2023). Furthermore, irrespective of the diet, SA or WW, the networks were more complex under dry-wet conditions than under continuous moisture, suggesting that the variable water availability also favored community interactions with different keystone taxa. The promoted microbial cooperation was suggested being important for survival under environmental stresses (Banerjee et al., 2019), which may lead to a higher resilience of the cast microbial communities towards the stresses of drying periods. Overall, considering that the dry-wet cycles applied in this study reflected actual field conditions more closely than the continuous moisture conditions, we conclude that legacy effects of earthworm gut transit on soil microbiomes will typically extend beyond a vegetation period.

In summary our study shows that the earthworm gut transit increased the abundances of bacteria, and fungi, but not of archaea, thus confirming our first hypothesis only for two of the three microbial domains. It favored copiotrophic bacteria, most likely denitrifiers, especially Flavobacteriales which showed generation times as short as 2 h. Compared to a diet with a high C/N ratio, a lower C/N ratio supported stronger bacterial growth. We could also confirm that both structure and activity of the soil microbiome responded to gut transit and the cast ageing, as indicated at the DNA and RNA level. We also confirmed that the water-limitation during dry-wet cycles preserves cast microbiomes longer than under constant moisture, thus slowing down the ageing process. For both conditions, however, compositional changes of the prokaryotic microbiome triggered by the gut passage were tangible after 168 days. More complex prokaryotic networks were detected under N-limitation of substrates and periods of water-limitation, suggesting that these conditions were more favorable for microbial cooperation than competition.

In conclusion, this study demonstrates that earthworm soil interactions strongly affect the diversity and temporal dynamics of the soil microbiome beyond the time of a vegetation period. Legacy effects of earthworm activities should therefore be kept in mind when investigating the environmental variation of soil microbiomes in agroecosystems.

CRediT authorship contribution statement

Jingjing Yang: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Stefan Schrader:** Conceptualization, Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing. **Christoph C. Tebbe:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing – original draft, Writing – review & editing, Supervision.

Declaration of competing interest

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2023.109294.

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