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Molecular profiling of 16S rRNA genes reveals diet-related differences of microbial communities in soil, gut, and casts of *Lumbricus terrestris* L. (Oligochaeta: Lumbricidae)

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Abstract

Earthworms are important members of the soil macrofauna. They modify soil physical properties, soil organic matter decomposition, and thus regulate carbon and nitrogen cycling in soil. However, their interactions with soil microorganisms are still poorly understood, in particular the effect of gut passage on the community structure of ingested microorganisms. Moreover, it is still unsolved, if earthworms, like many other soil-feeding invertebrates, possess an indigenous gut microbial community. Therefore, we investigated the bacterial and archaeal community structure in soil (with and without additional beech litter), gut, and fresh casts of *Lumbricus terrestris*, an anecic litter-feeding earthworm, by means of terminal-restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA gene fragments. Ecological indices of community diversity and similarity, calculated from the T-RFLP profiles, revealed only small differences between the bacterial and archaeal communities in soil, gut, and fresh casts under both feeding conditions, especially in comparison to other soil-feeding invertebrates. However, multivariate statistical analysis combining multidimensional scaling and discriminant function analysis proved that these differences were highly significant, in particular when the earthworms were fed beech litter in addition. Because there were no dominant gut-specific OTUs detectable, the existence of an abundant indigenous earthworm microbial community appears unlikely, at least in the midgut region of *L. terrestris*.

Keywords: Earthworm; Bacteria; Archaea; T-RFLP analysis; Multivariate statistical analysis

1. Introduction

At the end of the 19th century, Charles Darwin was one of the first who recognized and described the great importance of earthworm activity for the quality of soils [1]. Today, it is widely accepted that earthworms, like other representatives of the soil macrofauna, modify soil physical properties, affect soil organic matter decomposition, and thus regulate carbon and nitrogen cycling in soil [2–4]. A major part of the beneficial effects of earthworm activity on soil properties is contributed to interactions with soil microorganisms (for reviews see [5,6]). However, these interactions are still poorly understood, including the effect of gut passage on the community structure of ingested soil microorganisms.

Earthworm guts may be considered as favorable habitats for bacteria, because several studies showed increased microbial numbers in the guts versus the soil, in which earthworms were living [7–11]. In contrast, an increase in microbial biomass by the gut passage was not always observed [12–14]. While some early studies proposed that the earthworm gut microbial community is qualitatively not much different from the microbial community in the surrounding soil [7,8], later studies

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found significant differences for selected phylogenetic groups or functional guilds of microorganisms, e.g., *Proteobacteria* [15], *Actinobacteria* [16], denitrifiers [17,18] or cellobiose utilizers [19]. Compared to bacteria, only little attention was paid to the archaeal community in earthworm guts or casts [17,18,20]. Up to now only a few studies claimed that earthworms (including *Lumbricus terrestris*) have an indigenous gut microbial community [21,22], which is typical for other soil-feeding invertebrates, e.g., soil-feeding termites [23,24].

In this study we compared the overall community structure of *Archaea* and *Bacteria* in soil, gut, and fresh casts of *L. terrestris* by means of terminal-restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA gene fragments. This molecular fingerprinting technique (for reviews see [25,26]) was recently applied to investigate the microbial community structure in soil and gut of soil-feeding termites [27,28] and humivorous beetle larvae [29]. We chose *L. terrestris*, a large, deep burrowing (anecic sensu Bouché [30]) earthworm as model organism, because this species dominates earthworm biomass in many temperate ecosystems and strongly affects organic matter transformation and soil development [31,32]. We investigated if the passage through the gut of *L. terrestris* actually changes the soil microbial community and if there are indications for a gut-specific microbial community. Moreover, we tested if addition of beech litter to the soil affects the potential differences in microbial community structure between soil, gut, and casts of this earthworm species.

2. Materials and methods

2.1. Earthworms and feeding conditions

Adult earthworms (*L. terrestris* L.) were obtained by formalin extraction in an oak-beech forest 20 km south of Darmstadt (''Jägersburger Wald'', Germany), washed twice with distilled water, and kept at 5 °C in containers with soil (see below). Earthworms were shifted to 20 °C one week before experiments.

Soil and beech leaf litter were obtained from the ''Göttinger Wald'' plateau, a submontane beechwood forest on limestone, about 8 km east of Göttingen (Germany), with a canopy layer consisting almost exclusively of 115–120 year-old beech (*Fagus sylvatica*) trees (for further details see [33]). Soil (rendzina) was taken from the top 10 centimetres in October 2001, sieved (<4 mm) and frozen (−28 °C) until the experiment was started. Overwintered beech litter was collected from the soil surface at the same time, air-dried, and mechanically fragmented to pieces <4 mm.

The feeding experiment was set up with three independent replicate microcosms per feeding condition, resulting in three replicate soil, gut, and cast samples per feeding condition, respectively. The experiment was conducted in two vertically arranged vessels, each consisting of two transparent planar PVC sheets (650×310 mm) separated by solid non-transparent PVC strips (10-mm thick) on either side, at the bottom, and a pierced one on the top. Each vessel was separated into three compartments (650×100 mm) by further plastic-strips. These six vessel compartments were filled with soil at a level of 500 mm. Soil bulk density was adjusted to 0.70 kg dry weight l^{-1} and moisture content was kept constant at 70% (dry wt). Three randomly chosen vessel compartments were supplemented with 1 g beech litter, placed on top of the soil ([+]-beech litter treatment). Five days before the earthworms were placed in the vessels, they were kept on wet filter paper to eliminate their gut content. One *L. terrestris* specimen (mean body mass 3.2 ± 0.5 g) was put into each compartment and the vessels were subsequently incubated for three days at 20 °C in the dark.

2.2. Sampling and DNA extraction

After incubation, all six earthworms were killed, washed, and frozen at −20 °C. The litter added on top of the three [+]-beech litter microcosms had totally disappeared during incubation, indicating consumption by the earthworms. Fresh casts (0–3 days old) were carefully collected in each compartment and also frozen. Soil samples in the [−]-beech litter treatment were taken at least 50 mm away from the nearest burrow, where they had not been affected by earthworm activity. In the [+]-beech litter treatment, three soil samples carefully mixed with 1 g of beech litter each and incubated for 3 days under the same conditions as the vessels, served as control [12]. Earthworms were dissected with sterile instruments. The gut behind the gizzard was equally divided into three parts and the middle one was used for DNA extraction. The used gut section approximately corresponded to the midgut sections A and B as depicted in [34], which were shown to possess many potential attachment sites for microorganisms [21].

DNA was extracted from soil, gut (wall plus content), and cast samples (ca. 0.5 g) following a bead-beating protocol for cell disruption as described by Henckel et al. [35]. The protocol was slightly changed as DNA was purified from the supernatant with phenol/chloroform/isoamylalcohol (25:24:1) instead of ammonium acetate, followed by consecutive isopropanol and ethanol precipitation. Humic substances were removed with polyvinylpolypyrrolidone-filled spin-columns as described previously [29].

2.3. T-RFLP analysis

16S rRNA genes were specifically amplified using the primer combination of 6-carboxyfluorescein (FAM)-la-

beled primer 27f (5′-AGA-GTT-TGA-TCC-TGG-CTC-AG-3′ [36] and 907r (5′-CCG-TCA-ATT-CCT-TTR-A GT-TT-3′ [37] for *Bacteria* and Ar109f (5′-ACK-GCT-CAG-TAA-CAC-GT-3′ [38] and FAM-labeled Ar915r (5′-GTG-CTC-CCC-CGC-CAA-TTC-CT-3′ [39] for *Archaea*. The standard reaction mixture contained, in a total volume of 50 μl, 1× PCR buffer II (Applied Biosystems, Weiterstadt, Germany), 1.5 mM $MgCl_2$, 50 μM of each of the four deoxynucleoside triphosphates (Amersham Pharmacia Biotech, Freiburg, Germany), 0.5 μM of each primer (MWG Biotech, Ebersberg, Germany), 10 ng of bovine serum albumin (Roche, Mannheim, Germany), and 1.25 U of AmpliTaq DNA polymerase (Applied Biosystems). In addition, 1 μl of a 1:100 dilution of DNA extract (soil, gut or casts) was added as template. All reactions were prepared at 4 °C in 0.2 ml reaction tubes to avoid non-specific priming. Amplification was started by placing the reaction tubes immediately into the preheated (94 °C) block of a Gene Amp 9700 Thermocycler (Applied Biosystems). The standard thermal profile for the amplification of 16S rRNA genes was as follows: initial denaturation (94 °C, 3 min), followed by 32 (*Bacteria*) or 35 (*Archaea*) cycles of denaturation (94 °C, 30 s), annealing (52 °C, 45 s), and extension (72 °C, 90 s). After terminal extension (72 °C, 5 min), samples were stored at 4 °C until further analysis. Aliquots (5 μl) of 16S rRNA gene amplicons were analyzed by gel electrophoresis on 1% agarose gels and visualized after staining with ethidium bromide. PCR products were purified using the MinElute PCR Purification Kit (Qiagen, Hilden, Germany).

Prior to digestion, amplicon concentrations were determined photometrically. DNA (75 ng), 2.5 U of restriction enzyme (*Msp*I [bacterial amplicons] or *Alu*I [archaeal amplicons]; Promega, Mannheim, Germany), 1 μl of 10× incubation buffer, and 1 μg of bovine serum albumin were combined in a total volume of 10 μl and digested for 3 h at 37 °C. Preliminary tests with *Bst*UI and *Hae*III as restriction enzymes, performed with a subset of samples, revealed results highly comparable to those obtained with *Msp*I regarding the similarity of soil, gut, and casts samples. To allow for a comparison of the results obtained with *L. terrestis* to results obtained recently for humivorous beetle larvae [29] and soil-feeding termites [28], we chose *Msp*I as restriction enzyme. Fluorescently labeled T-RFs were size separated on an ABI 373A automated sequencer (Applied Biosystems) using an internal size standard (GeneScan-1000 ROX; Applied Biosystems). T-RFLP electropherograms were analyzed with GeneScan 2.1 software (Applied Biosystems).

2.4. Statistical analysis

Prior to statistical analysis all bacterial and archaeal T-RFLP electropherograms (starting from 50 bp to exclude T-RFs caused by primer dimers) were normalized to identical total peak heights, respectively, using an iterative method described by Dunbar et al. [40], where all T-RFs with a height less than 50 relative fluorescence units were omitted. All subsequent calculations were performed with major T-RFs, arbitrarily defined as those with a relative peak height of $\geqslant 1.5\%$ of the total electropherogram peak height. To ensure that the relative heights of all major T-RFs equal 100%, they were normalized to the total peak height of all major T-RFs per electropherogram. As the apparent size of identical T-RFs can vary in a range of 1–2 bp among different gels and/or lanes of the same gel, major T-RFs similar in size of ±1–2 bp were summarized to operational taxonomic units (OTUs). Applying the above-mentioned 1.5% threshold for the definition of major T-RFs, ca. 30 different bacterial and archaeal OTUs each could be created from the analyzed T-RFLP profiles, which were used for the statistical analysis of the different samples.

For the calculation of ecological indices, OTUs were treated as species and their relative height served as a measure of relative abundance. Shannon–Wiener indices and (Shannon-) evenness [41] were used to compare diversity among the different samples and treatments. Evenness values were calculated using the natural logarithm of the number of OTUs per single electropherogram. To describe the pairwise similarity of microbial communities, e.g., between soil and gut, Morisita indices of community similarity were used as described in [42]. Morisita indices range from 0 to 1, with 1 indicating complete (100%) identity of two communities. Differences in ecological indices between different samples and treatments were checked for statistical significance ($P < 0.05$) using non-parametric tests (Mann–Whitney, Kruskal–Wallis).

To compare the overall structure of bacterial and archaeal communities among the different compartments (soil, gut, and casts) and treatments (with and without beech litter) multidimensional scaling (MDS) and discriminant function analysis (DFA) were used, based on a scheme proposed by Puzachenko and Kuznetsov [43], previously applied for microfungal communities by Tiunov and Scheu [44]. In detail, a square matrix of non-parametric Gamma correlation (analogous to Kendall τ) was calculated from the relative frequencies of all bacterial and archaeal OTUs, respectively. This matrix was analyzed by multidimensional scaling, i.e., an ordination technique, which "rearranges" objects in a maximum nine-dimensional space, so as to arrive at a configuration that best approximates the observed distances. The number of meaningful dimensions was evaluated by comparing actual stress values, representing a measure for the loss of information when the data are fitted into the *n*-dimensional space, with the theoretical exponential function of stress. The coordinates of the samples in the *n*-dimensional space were

used for discriminant function analysis, with "compartment" (soil, gut, and casts) as a grouping variable. Squared Mahalanobis distances between group centroids and reliability of sample classification were determined. Typically, only two significant discriminatory axes were derived and, therefore, the results of DFA were graphically presented in two dimensions. For the interpretation of the discriminant axes with respect to the frequency of OTUs, linear correlations were calculated between the discriminant function scores for each sample and the relative OTU frequencies. All MDS and DFA calculations were performed using the STATISTICA (6.0) software package.

3. Results

Archaeal and bacterial 16S rRNA gene fragments were successfully amplified from six soil, six gut, and six cast DNA extracts, respectively (three extracts per feeding condition, each). All results are based on the analysis of normalized 16S rRNA gene fingerprints obtained from three independent replicate samples per compartment and feeding condition, respectively.

3.1. Archaeal community structure

The T-RFLP profiles of the archaeal community structure in soil, gut, and fresh casts of *L. terrestris* were similar, in particular without beech litter as additional food source for the earthworms (Fig. 1(a)). None of the calculated ecological indices (Table 1) revealed significant differences in the community structure among soil, gut or casts under both feeding conditions. However, the addition of beech litter to the soil had a significant influence, when corresponding compartments were compared. Based on Shannon–Wiener and evenness values, litter significantly ($P < 0.05$) increased the archaeal diversity in soil and casts, but not in the gut. In contrast, litter did not affect community similarity. Morisita indices in the range of 0.92–0.96 indicated that the archaeal community was almost identical in soil, gut, and casts, when no litter was added. Addition of litter lowered the average similarity of the archaeal commu-

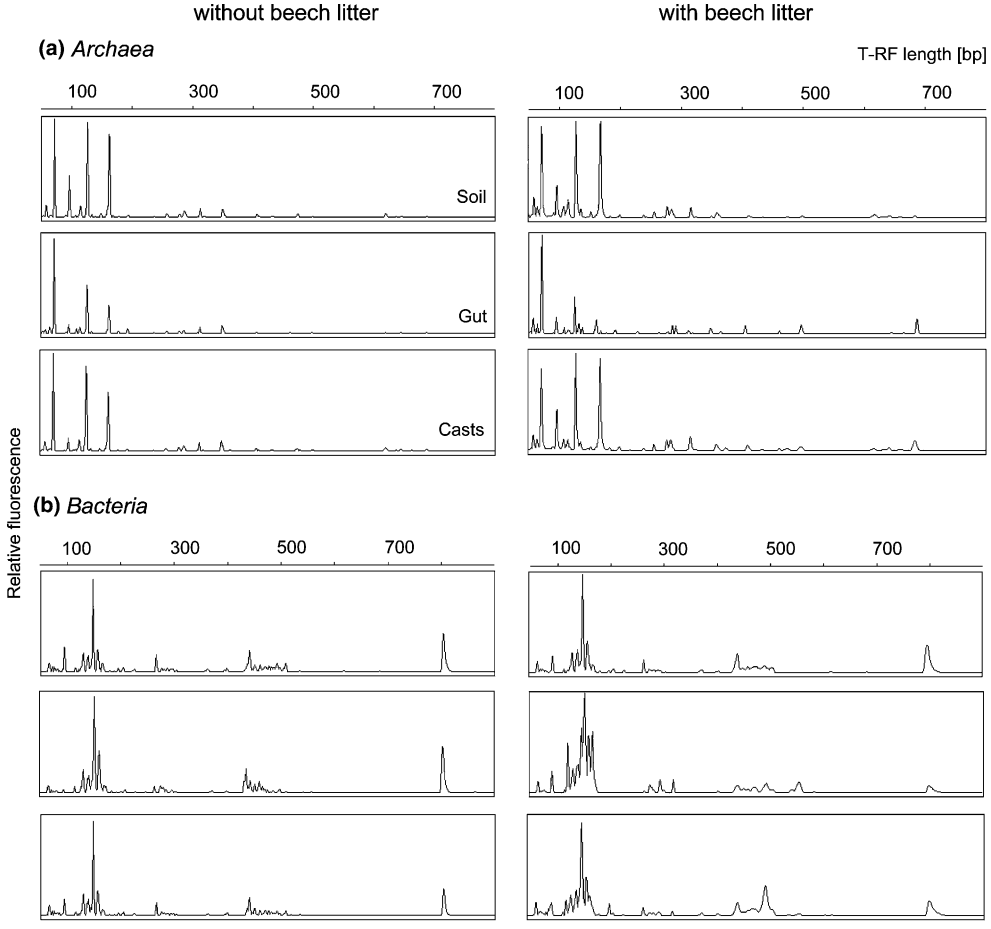

Fig. 1. Exemplary T-RFLP profiles of archaeal (a) and bacterial (b) 16S rRNA genes amplified from DNA extracts of soil, gut, and fresh casts of two *Lumbricus terrestris* earthworms. One was kept for three days without litter (left side), the other one with additional beech litter (right side) in its soil, respectively. *Alu*I (*Archaea*) and *Msp*I (*Bacteria*) were used for restriction digest. Note that in total three soil, gut, and cast samples each were investigated per feeding condition, resulting in 18 archaeal and 18 bacterial fingerprints to be compared (of which only six each are depicted here).

Table 1
Diversity and similarity indices characterizing the archaeal community structure in soil, gut, and fresh casts of *Lumbricus terrestris* under two feeding conditions (FC; with [+] and without [–] additional beech litter), based on T-RFLP analysis of 16S rRNA gene fragments

Index	FC	Soil	Gut	Casts
Diversity (Shannon–Wiener)	–	1.68 ± 0.21a	1.89 ± 0.17a	1.61 ± 0.19a
	+	2.12 ± 0.05b	2.03 ± 0.15ab	2.21 ± 0.06b
Evenness	–	0.76 ± 0.02a	0.77 ± 0.04a	0.73 ± 0.04a
	+	0.82 ± 0.01b	0.79 ± 0.02ab	0.83 ± 0.01b
		Soil:gut	Soil:casts	Gut:casts
Similarity (Morisita)	–	0.92 ± 0.02a	0.96 ± 0.01a	0.93 ± 0.01a
	+	0.77 ± 0.08a	0.98 ± 0.01a	0.80 ± 0.05a

Values are means ± SEM ($n = 3$, for each compartment and treatment). Different letters indicate significant ($P < 0.05$) differences.

nity in the guts to the communities in soil and casts, however, this change was not statistically significant.

In contrast to the ecological indices, the combination of MDS and DFA detected significant differences between the investigated compartments (soil, gut, casts) under both feeding conditions. Multivariate statistical analysis of all archaeal OTU frequencies resulted in two discriminant axes (Fig. 2). OTUs showing a significant correlation to one of these axes, i.e., OTUs responsible for the separation of the compartments, are given in Table 2. Without beech litter, T-RFLP profiles of earthworm guts were significantly separated from the soil profiles, but not from the cast profiles (Fig. 2; for squared Mahalanobis distances and levels of significance for discrimination of the different compartments see Table 3). There was also no significant discrimination between cast and soil profiles. The addition of litter to the soil markedly increased the differences in the archaeal community structure. Under this feeding condition, all investigated compartments were significantly discriminated from each other with the largest difference between earthworm guts and all other treatments. When the statistical analysis was expanded from major T-RFs (with a relative peak height of $\geqslant$1.5% of the total electropherogram peak height) to all T-RFs in the normalized electropherograms, casts were discriminated from the guts even without additional beech litter, however, casts with and without litter remained indiscriminate (data not shown).

To find possible indications for an indigenous earthworm microbial community, archaeal T-RFLP-profiles were screened for gut-specific OTUs, defined as occurring in the majority (at least 4 out of 6) of earthworms, i.e., under both feeding conditions, exclusively in the gut sections or at least in gut and cast profiles, but not in the soil profiles. However, not a single archaeal OTU (out of 29) fulfilled these requirements.

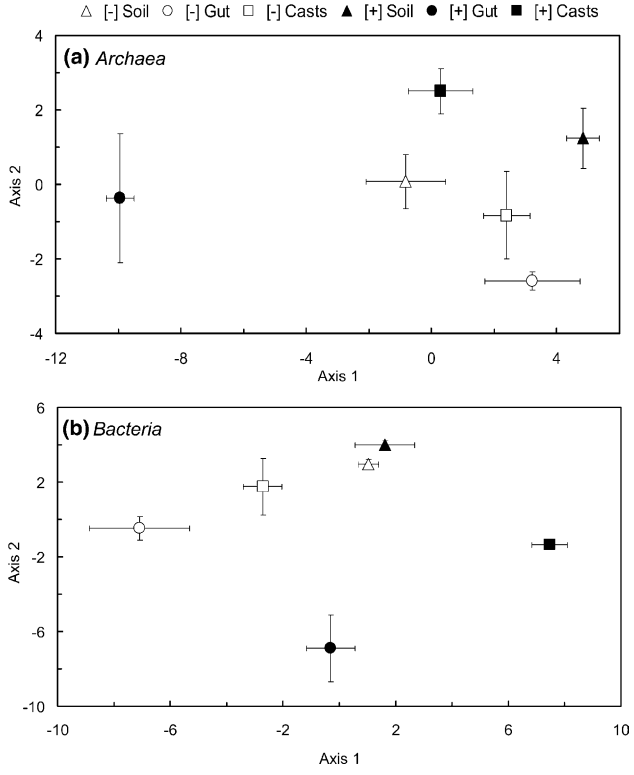

Fig. 2. Discriminant function analysis of the relative frequencies of archaeal (a) and bacterial (b) OTUs in T-RFLP profiles of 16S rRNA genes amplified from soil, gut, and fresh casts of *Lumbricus terrestris* earthworms kept for three days with ([+], closed symbols) and without ([–], open symbols) additional beech litter in their soil, respectively. Shown are group centroids of treatments with one standard deviation for axis 1 and axis 2. Each centroid is representing three independent replicates. In case of the archaeal OTUs, axis 1 accounts for 87.2% ($P < 0.0001$) of the variance, axis 2 for 9.6% ($P < 0.05$). In case of the bacterial OTUs axis 1 accounts for 56.5% ($P < 0.0001$) of the variance, axis 2 for 37.4% ($P < 0.0001$).

3.2. Bacterial community structure

Like the archaeal profiles, the bacterial profiles for all compartments were similar, particularly without additional litter (Fig. 1(b)). Evenness values and Morisita indices of community similarity were not significantly different, when soil, guts, and casts were compared for both feeding conditions (Table 4). With additional litter, OTU diversity in the casts, based on Shannon–Wiener indices, was significantly ($P < 0.05$)

Table 2
Relative percentile frequencies (means ± SEM; $n = 3$, for each compartment and treatment) of archaeal OTUs (bp) with a linear correlation (*r*-values) to one of the two discriminant axes (see Fig. 2)

OTU	Without additional litter			With additional litter			Correlation	
	Soil	Gut	Casts	Soil	Gut	Casts	Axis 1	Axis 2
108	n.d.	2.8 ± 0.8	0.5 ± 0.5	3.9 ± 0.6	0.7 ± 0.7	3.0 ± 0.2	−0.449	−0.650**
114	3.0 ± 1.8	3.5 ± 0.5	2.8 ± 0.5	5.2 ± 1.5	n.d.	3.3 ± 0.6	−0.552*	−0.136
126	32.6 ± 4.6	22.7 ± 1.2	34.3 ± 4.9	21.6 ± 1.1	13.0 ± 0.5	22.0 ± 0.4	−0.492*	0.221
274	n.d.	n.d.	n.d.	2.7 ± 0.2	n.d.	2.4 ± 0.1	−0.245	−0.742***
286	1.2 ± 0.6	n.d.	0.6 ± 0.6	1.9 ± 0.1	3.9 ± 1.4	2.9 ± 0.2	0.705**	−0.330
409	n.d.	n.d.	n.d.	n.d.	3.1 ± 0.7	n.d.	0.848***	0.005
498	n.d.	n.d.	n.d.	n.d.	2.9 ± 1.5	n.d.	0.708**	−0.119
687	n.d.	n.d.	n.d.	n.d.	5.5 ± 0.9	2.5 ± 0.2	0.832***	−0.196
800	n.d.	n.d.	n.d.	n.d.	0.8 ± 0.8	n.d.	0.638**	−0.146

n.d., OTU not detected.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

Table 3
Squared Mahalanobis distances between group centroids and reliability of discrimination based on relative frequencies of archaeal and bacterial (in italics) OTUs

	[−] Soil	[−] Gut	[−] Casts	[+] Soil	[+] Gut	[+] Casts
[−] Soil	0	44.8*	21.2	58.8**	134.0***	19.0
[−] Gut	*124.5***	0	7.4	26.2	267.3***	52.0**
[−] Casts	*28.3**	*43.7**	0	16.3	229.7***	24.6
[+] Soil	*19.7*	*152.6****	*50.4***	0	331.3***	33.6*
[+] Gut	*153.6****	*137.0****	*126.0****	*187.8****	0	169.5***
[+] Casts	*97.1***	*319.8****	*174.2****	*106.7****	*143.1****	0

[−], without additional beech litter; [+], with additional beech litter.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

higher compared to gut and soil. When corresponding compartments were compared, the addition of beech litter had a significant influence, in particular on the diversity indices. Shannon–Wiener indices increased significantly ($P < 0.05$) in all compartments, when litter was added to the soil. The evenness decreased in the soil, while it remained unaffected in guts and casts. Community similarity between soil and guts (94%), determined by means of Morisita indices, significantly ($P < 0.05$) decreased to 77% when the earthworms ingested additional litter.

While the ecological indices revealed only small differences between the investigated compartments, the multivariate statistics approach again was highly effective in discrimination. Statistical analysis of the frequencies of all bacterial OTUs resulted in five discriminant axes, two of which are depicted in Fig. 2. OTUs with a significant correlation to one of these two

Table 4
Diversity and similarity indices characterizing the bacterial community in soil, gut, and fresh casts of *Lumbricus terrestris* under two feeding conditions (FC; with [+] and without [−] additional beech litter), based on T-RFLP analysis of 16S rRNA gene fragments

Index	FC	Soil	Gut	Casts
Diversity (Shannon–Wiener)	−	2.13 ± 0.09a	2.11 ± 0.05a	2.17 ± 0.03a
	+	2.37 ± 0.02b	2.32 ± 0.01b	2.51 ± 0.02c
Evenness	−	0.89 ± 0.01a	0.86 ± 0.01a	0.88 ± 0.02a
	+	0.86 ± 0.01b	0.89 ± 0.01ab	0.90 ± 0.01ab
		Soil:gut	Soil:casts	Gut:casts
Similarity (Morisita)	−	0.94 ± 0.01a	0.96 ± 0.02a	0.94 ± 0.01a
	+	0.77 ± 0.07b	0.91 ± 0.01ab	0.89 ± 0.03ab

Values are means ± SEM ($n = 3$, for each compartment and treatment). Different letters indicate significant ($P < 0.05$) differences.

Table 5
Relative percentile frequencies (means ± SEM; $n = 3$, for each compartment and treatment) of bacterial OTUs (bp) with a linear correlation (*r*-values) to one of the two discriminant axes (Fig. 2)

OTU	Without additional litter			With additional litter			Correlation	
	Soil	Gut	Casts	Soil	Gut	Casts	Axis 1	Axis 2
91	6.5 ± 0.4	n.d.	3.7 ± 0.7	5.0 ± 0.3	2.1 ± 1.2	4.6 ± 1.1	0.636**	0.469*
121	n.d.	n.d.	n.d.	n.d.	7.2 ± 1.4	3.8 ± 0.1	0.347	−0.895***
147	5.1 ± 0.4	5.3 ± 1.1	5.1 ± 1.3	3.0 ± 0.1	11.5 ± 2.0	6.4 ± 0.8	0.012	−0.904***
153	27.3 ± 0.7	26.9 ± 0.8	27.8 ± 0.5	31.3 ± 0.8	20.6 ± 2.6	25.4 ± 1.3	−0.049	0.860***
160	13.2 ± 0.9	19.9 ± 1.3	14.8 ± 0.8	10.0 ± 0.1	11.9 ± 1.2	8.9 ± 0.3	−0.855***	0.066
165	n.d.	n.d.	n.d.	0.9 ± 0.9	13.8 ± 3.3	10.0 ± 0.6	0.462	−0.848***
199	n.d.	n.d.	n.d.	n.d.	n.d.	4.8 ± 0.9	0.722**	−0.157
266	4.8 ± 0.2	n.d.	3.2 ± 0.6	4.0 ± 0.1	n.d.	2.3 ± 0.2	0.393	0.770***
295	n.d.	n.d.	n.d.	n.d.	2.0 ± 1.1	n.d.	−0.015	−0.751***
439	7.0 ± 0.8	9.0 ± 1.3	6.4 ± 0.7	5.9 ± 0.3	1.7 ± 0.9	3.4 ± 0.2	−0.594**	0.608**
450	n.d.	1.8 ± 0.9	0.7 ± 0.7	0.6 ± 0.6	0.6 ± 0.6	n.d.	−0.583*	−0.090
471	n.d.	2.9 ± 0.1	0.9 ± 0.9	2.0 ± 0.1	n.d.	0.6 ± 0.6	−0.467*	0.288
488	n.d.	n.d.	n.d.	n.d.	n.d.	2.1 ± 1.1	0.604**	−0.127
496	1.7 ± 0.9	n.d.	n.d.	2.2 ± 0.0	2.4 ± 0.5	5.7 ± 1.0	0.840***	−0.247
557	n.d.	n.d.	n.d.	n.d.	2.3 ± 1.4	n.d.	−0.027	−0.712**
798	15.6 ± 2.9	11.9 ± 1.1	11.3 ± 4.4	7.7 ± 0.8	1.6 ± 0.9	4.0 ± 0.3	−0.353	0.617**

n.d., OTU not detected.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

axes, i.e., OTUs that are responsible for the separation of the compartments, are given in Table 5. Under both feeding conditions earthworm guts, casts and soil were significantly discriminated. However, the discrimination was more pronounced, when litter was added (Fig. 2; for squared Mahalanobis distances and levels of significance for discrimination of the different compartments see Table 3). Beech litter significantly changed the bacterial community structure in the gut and cast compartments with the gut separated from all other treatments along the second axis. Because we did not aim to investigate the effect of litter addition on the soil microbial community structure, it was not attempted to discriminate the soil samples by lowering the threshold level for major T-RFs.

Also the bacterial T-RFLP-profiles were screened for gut specific OTUs. However, as in case of the archaea, none out of 27 OTUs occurred exclusively in the majority of earthworm guts or casts but not in the soil samples.

4. Discussion

In this study, we analyzed the overall bacterial and archaeal community structure in soil, gut, and fresh (0–3 days old) casts of an earthworm (*L. terrestris*) under different feeding conditions using a molecular, culture-independent fingerprinting technique. Using multivariate statistical analysis we found a significant influence of gut passage and diet (with and without beech litter) on the composition of the ingested microbial community.

4.1. Methodological considerations regarding T-RFLP analysis

T-RFLP analysis is a PCR-based method, which can be biased (e.g. [45,46], for a review see [47]). Therefore, the amplicon pools obtained from the different samples did not necessarily reflect the quantitative composition of the underlying microbial communities. However, studies exist, showing that PCR-T-RFLP may adequately reflect the relative composition of functional [48] or 16S rRNA genes [49] in model communities. Regarding soil type and earthworm species, the samples investigated in this study were quite similar, as indicated by the highly similar profiles, and they were all investigated with the same PCR-conditions. Thus, all samples were probably affected equally by a potential PCR-bias, i.e., the comparison of the different fingerprints is probably not affected by this kind of bias. The resolution limit of T-RFLP analysis is another important point to mention. The different OTUs, which are the basis for the comparison of the investigated compartments, probably do not represent different microbial species but heterogeneous groups of species sharing the same restriction sites. However, considering not only the presence or absence of OTUs but also their relative frequency should provide a good basis for comparing microbial community fingerprints. In other words, if two fingerprints match in presence and frequency of different OTUs, this is indicative of a high similarity of the underlying amplicon pools. A highly comparable PCR-T-RFLP approach as used in this study, using the Morisita index as a measure of fingerprint similarity, has

just recently proven to be suitable to reveal marked differences in microbial community structure between ingested soil and the different gut sections of humivorous beetle larvae [29] and soil-feeding termites [28]. Despite the limited resolution of T-RFLP analysis, several bacterial and archaeal T-RFs were found to occur exclusively in the gut but not in the food soil profiles of these animals, indicating indigenous populations of microorganisms.

4.2. Gut specificity of the intestinal microbial community of L. terrestris

Based on Morisita community similarity indices, the archaeal and bacterial community of the earthworm gut was very similar (92–96% similarity) to the communities in soil and casts, in particular without litter as food source. The addition of beech litter resulted in decreased similarity values, particularly between gut and soil (77% similarity), but this decrease was statistically significant only for the bacterial community. Our findings corroborate the results of other studies that most (if not all) bacteria in the earthworm gut originate from the ingested soil [7,8,20,34,50], and are suitable to extend this hypothesis also onto the archaeal community in the earthworm gut. So far, the only indication for an indigenous gut microbial community in a *Lumbricus* earthworm species came from an electron microscopy study [21] reporting on rod-shaped cells specifically attached to the hindgut wall of 4 out of 10 examined *L. terrestris* specimens. We assumed that a dominant indigenous earthworm microbial community should result in gut-specific or (allowing some wash-out) at least gut- and cast-specific OTUs that do not appear in the soil profiles and are independent of the food, consumed by the earthworms. However, none of ca. 30 investigated archaeal and bacterial OTUs each fulfilled these assumptions. This finding, together with the high Morisita similarity values obtained for the comparison of gut and soil profiles, argues against an abundant indigenous bacterial or archaeal earthworm microbial community, at least in the midgut section of *L. terrestris*. However, due to the limited resolution of T-RFLP analysis it cannot be fully excluded that gut-specific groups of microorganisms could not be differentiated from soil-derived microorganism with the same restriction sites and/or that were discriminated by the used general primer systems. The lack of a dominant indigenous microbiota in the earthworm gut is contrary to other soil-feeding invertebrates, e.g., humivorous beetle larvae [29] or soil-feeding termites [28] and may indicate that intestinal microbes in the earthworms are rather food than symbionts aiding digestion. However, compared to fungi and protozoa, soil bacteria are supposed to be of little importance as food source for earthworms [6]. The absence of a specific gut microbial community might be attributed to the rather simple, tubular morphology of the earthworm gut [31], lacking any distinct "fermentation chambers" characteristic for the intestinal tract of scarabaeid beetle larvae and soil-feeding termites [51–53]. The gut of *L. terrestris* possesses morphological features allowing a differentiation into a fore-, mid- and hindgut, and the typhlosole fold to increase the gut surface [21,31], but special attachment structures for microorganisms are lacking. In contrast, the hindgut of scarabaeid beetle larvae contains a great number of featherlike chitin-structures, densely covered with a biofilm of microorganisms [54]. These structures might be one reason for the existence of a special gut microbial community in these larvae, despite the fact that they exchange their gut content ca. twice a day [53], which is in the range of earthworms such as *L. terrestris* [8].

4.3. Effect of gut passage and litter addition on community structure of ingested microbes

The multivariate statistics approach combining MDS and DFA was highly effective in analyzing T-RFLP data and appears to be a promising tool for further fingerprint studies, for which appropriate methods of analysis are currently discussed [55]. The bacterial community in the earthworm guts was significantly discriminated from the communities in soil and casts, which also were significantly different from each other. The addition of litter markedly sharpened these differences. The influence of litter might be represented by the discrimination along axis 2 (Fig. 2(b)), along which all samples without litter were largely similar. Hypothetically, axis 1 represents the influence of gut passage, since soil, gut, and cast samples without litter are effectively separated along axis 1; this hypothesis is further supported by the fact that the casts displayed an intermediate position between the soil and gut samples. However, in the presence of litter, this discrimination along axis 1 was not evident, rather it appears that additional litter (axis 2) has a much stronger impact on the intestinal community than the gut passage (axis 1) per se. In case of the archaea, the influence of litter addition was so strong that in principle all data could be grouped into two categories: gut with litter and all others. Significant differences between the microbial community composition of different compartments detected with the multivariate statistics approach are not contradictory to the results obtained with the ecological indices, indicating a high similarity of all compartments under both feeding conditions. The differences in microbial community structure between soil, earthworm gut, and casts were obviously beyond the resolution limit of the used ecological indices. However, the differences in the overall community structures were rather small, but consistent among the

replicate samples, and therefore significant, which became obvious, when analyses were performed with more sophisticated methods.

Several reasons for differences between the microbial community structure of soil, earthworm gut, and casts are possible: (A) selective feeding of the earthworm on hot spots of microbial life [11]; (B) anoxic conditions in the gut lumen [34] favoring microorganisms able to grow anaerobically [19]; (C) proliferation of microorganisms effectively exploiting the favorable physicochemical gut conditions [20,34]; (D) differential lysis of microbes by digestive enzymes secreted by the earthworm [6]; (E) inhibition of bacteria by inhibitory substances secreted by other bacteria [20,56]. The addition of beech litter to the soil markedly increased the differences between the investigated compartments for both bacteria and archaea, which is most likely due to preferential feeding of the earthworms on the supplemented litter and associated microorganisms, since *L. terrestris* is a litter-feeding earthworm species, preferentially consuming litter in a mixture with mineral soil [57]. This hypothesis is corroborated by four archaeal and three bacterial OTUs occurring exclusively in the litter-supplemented gut and cast, but not in the soil samples. Ingested litter might also enhance some of the above-mentioned processes with influence on the microbial community structure, because it means an additional input of nutrients into the gut possibly stimulating the secretion of digestive enzymes by the earthworm. For endogeic *Hormogaster elisae* earthworms, it was suggested that at least the amount of mucus added to the gut increases with increasing content of organic matter in the soil [58]. Pure soil was shown to pass the gut of burrowing *L. terrestris* within 11–12 h, however, the time span of gut passage was prolonged to about 20 h, when the animals fed on additionally provided organic matter mixed with soil [8]. This means, additional litter could have increased the effect of gut passage also by prolonging the time span the microbial community was exposed to the gut conditions.

We conclude that the midgut microbial community of *L. terrestris* is largely soil- or food-derived, and that significant differences in community composition between soil, earthworm midguts, and casts probably do not result from a truly indigenous gut microbial community but rather from selective feeding of *L. terrestris* and the physicochemical conditions in its gut, possibly inducing changes in the community composition of ingested microorganisms.

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