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Trophic transfer of fatty acids from gut microbiota to the earthworm *Lumbricus terrestris* L

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Abstract

The diet of earthworms includes soil organic matter, soil microbes and other microfauna, but the relative contribution of these dietary components to earthworm nutrition is not well known. Analysis of fatty acid (FA) profiles can reveal trophic relationships in soil food webs, leading to a better understanding of the energy and nutrient flows from microbiota to earthworms. The objective of this study was to determine the origin of FAs assimilated by the earthworm *Lumbricus terrestris* L. We analysed the pattern of FAs in: (i) the bulk soil, (ii) soil in the earthworm gut, (iii) the absorptive tissue of the earthworm gut wall, and (iv) the muscular layers of the earthworm body wall. Multivariate analyses performed on the FA profiles suggest that the microbial community in the earthworm gut differs from that in bulk soil. Diverse bacterial and fungal derived FAs, which earthworms cannot synthesize, were found in the earthworm gut wall and body wall, and in the neutral lipids (storage lipids) of the gut wall. The major compounds isolated were 20:4ω6, 20:5ω3 and 18:2ω6, followed by the monoenoic 18:1ω7 and 18:1ω9c, and the saturated 18:0. The microbial FA assemblage in the gut wall resembled the gut soil more than the bulk soil, and the body wall of *L. terrestris* showed the same microbial derived FA pattern as the gut wall, although at reduced concentrations. We propose the existence of a specific microbial community in the earthworm gut that provides FAs to the earthworm. It appears that *L. terrestris* may derive more of its energy and nutrients from gut specific microbiota than from microbiota already present in the ingested soil, based on the trophic relationships revealed through FA analysis.

Keywords: Fatty acids; Gut microbial community; Food web; Earthworm; Trophic transfer

1. Introduction

Earthworms are well known for their proximate effects on soil respiration, organic matter decomposition and water infiltration, and for their contribution to large-scale soil processes such as soil fertility and pedogenesis (e.g. Edwards and Bohlen, 1996; Domínguez et al., 2004; Wolters, 2000). Many of the ecosystem-level functions attributed to earthworms result from biological interactions with microbes and soil fauna living in structures created by earthworms, namely burrows, middens and casts, as well as with the microbiota that inhabit the earthworm gut. There is growing evidence that earthworm gut microbiota have a central role in the digestion of organic substrates (Horn et al., 2003), but further studies are needed to elucidate such a relationship. In the other hand, the contribution of soil microbes and microfauna such as protozoa and nematodes (Domínguez et al., 2003) to the earthworm diet, i.e. their position in the soil food web relative to earthworms, remains unresolved too.

Since trophic transfer of intact lipids was first demonstrated in microbial-based tritrophic cascades (Ederington et al., 1995), FA and lipid signature biomarkers have been used quite widely to reveal trophic relationships in marine and freshwater food webs, sometimes in combination with isotope ratio mass spectrometry (Pond et al., 1997a, b;

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Boschker et al., 1998). Biosynthetic pathways that produce FAs are relatively group-specific, although interspecific variation in the cellular FA composition arises due to differences in the diet and metabolic activity of individuals, as well as in environmental conditions (Tunlid and White, 1990).

Much more is known about lipid metabolism and trophic transfer of FAs in aquatic food webs than soil food webs. Some singularities have been detected in the lipid metabolism of soil fauna, such as the unexpected existence of a complete range of desaturases and elongases in nematodes (Watts and Browse, 2002), and the absence of cholesterol synthesis in insects and earthworms (Wootton and Wright, 1962; Chamberlain et al., 2004). Besides, heterotrophic food webs, such as those involving soil microfauna, show complex multilevel relationships. Nevertheless, the lipid composition of nematodes and collembola has been used to confirm their diets and verify their trophic relations in soil food webs (Chen et al., 2001; Ruess et al., 2002, 2004, 2005; Chamberlain et al., 2004; Haubert et al., 2004). However, the use of lipid signature biomarker analysis for tracing trophic relationships in soil food webs is still developing. Better knowledge of the FA patterns in soil fauna and the factors affecting the trophic transfer of FAs within soil foodwebs is needed.

The objective of this study was to study the FA profiles present in earthworm tissues and to determine their origin. We studied the incorporation of FAs from the bulk soil and the gut soil into the absorptive tissue of the gut wall and the muscular layers of the body wall in the anecic earthworm *L. terrestris*.

2. Materials and methods

2.1. Experimental design and sampling

Adult *Lumbricus terrestris* L. ranging from 5 to 10 g (fresh weight) were collected from a corn field at the Macdonald Research Farm, Ste-Anne-de-Bellevue, Quebec, Canada and transferred to 500 ml culture bins with the original soil (sieved, <2 mm mesh). Soil at the site was a Humic Gleysol (fine-silty, mixed, frigid Typic Endoaquent) with 400 g sand kg^{-1}, 160 g clay kg^{-1}, 18.1 g organic C kg^{-1} and pH 5.5. Earthworms were maintained at 17 ± 1 °C in dark without added food until analysis (about 10 days). After removing earthworms from the container, they were immediately placed in a Petri plate with ice for 10 min to prevent casting. They were rinsed with sterilized cold saline solution (0.9% NaCl), followed by brief rinsing with 50% ethanol solution, and then euthanized in hot water (65 °C) for 10–15 s. Earthworms were placed on an ice-cold glass stage and dissected aseptically under a stereomicroscope. A longitudinal incision was made along the body wall and the whole gut was meticulously removed from the earthworm body and inspected. If the gut wall was split, the entire sample was discarded because the muscular tissue of the body wall could have been contaminated with FAs from the gut lumen or gut wall. Next, the gut was dissected and soil from the intestinal tract (referred to as 'gut soil') was collected carefully with dissecting needles. The body wall and the gut wall were each rinsed separately five times with cold sterile saline solution. A composite sample of the gut soil, gut wall and body wall was made from two earthworms reared in the same culture bin to provide enough material for lipid extraction. Samples of bulk soil (5.34 ± 0.40 g, mean $\pm$ SEM) from the corresponding culture bins ($n=7$) were also collected. Gut soil samples were, on average, 526 ± 86 mg, while gut wall samples contained 98 ± 11 mg and the body wall samples had 242 ± 21 mg of material (all weights expressed on a dry weight basis). All samples were frozen immediately after collection and then freeze dried and stored at -20 °C until lipid extraction. An additional set of five culture bins with two earthworms each, maintained and dissected as above, were used to obtain earthworm tissue from the midgut and hindgut wall in order to study the FA composition of the neutral lipid fraction (NLFA).

2.2. Lipid extraction and fractionation

Total lipids were extracted with methanol and chloroform (2:1, vol/vol) using an Accelerated Solvent Extractor ASE200 (Dionex Corporation, Sunnyvale, CA, USA) according to Macnaughton et al. (1997). Cells were heated to 80 °C and brought to 8.28 MPa for 5 min, then subject to static extraction for 15 min. Three static cycles were conducted on each sample. Fatty acid methyl esters (FAMEs) were prepared by a mild alkaline methanolysis of the total lipidic extract following the standardized procedure proposed by White and Ringelberg (1998). This procedure is suitable only for transesterification of ester-linked FAs (EL-FAs) as it does not methylate free FAs nor destroy the cyclopropane rings (Kates, 1986; Grogan and Cronan Jr., 1997). The fraction of neutral lipids (NL) in samples of earthworm gut wall was isolated by solid phase extraction (White and Ringelberg, 1998). The total lipidic extract (obtained as above) was applied to a silicic column, the neutral lipids were collected after elution with chloroform and FAMEs prepared as above.

2.3. Quantification and identification of fatty acids

Total FAMEs from bulk soil, gut soil, gut wall and body wall were analysed in split mode (50:1) with a gas chromatograph (Hewlett Packard 6890) equipped with a Simplicity Wax capillary column (cross-linked PEG, Supelco 2-4326), Helium as carrier gas and a flame ionization detector. The oven temperature was initially set at 60 °C, then raised to 150 °C (10 °C min^{-1}) and held for 5 min after which it was raised by 3 °C min^{-1} to a final temperature of 230 °C and held for 20 min. Inlet and detector temperatures were 200 and 250 °C, respectively. Methyl-nonadecanoate (19:0) was used as an internal standard (IS) for quantification of FAMEs with

appropriate standard curves, resulting in a linear conversion factor of 0.62 ng unit^{-1} of peak area.

Neutral lipid FAs from the gut wall were analysed using a HP 6890 GC equipped with an Ultra-2 capillary column, H_2 as carrier gas and a flame ionization detector, followings the settings of the MIDI protocol (MIDI, Inc., Newark, Delaware, www.midi-inc.com).

Identification of peaks was performed by comparison of equivalent chain length (ECL) to those of known compounds in commercial standards (Supelco 37 Component FAME Mix cat.#47885-U; Supelco Bacterial Acid Methyl Esters cat.#47080-U; Matreya PUFA-2 cat.#1081; Matreya Bacterial Acid Methyl Esters CP Mix cat.#1114; Matreya *cis*-11-Hexadecenoic Acid cat.#1208 and Matreya 10-Methyloctadecanote cat.#1763), which allowed us to identify 53 FAMEs. The correct identification and quantification of the standards was cross-validated by duplicate analyses of a range of samples and standards in a certificated external laboratory (Laboratoire de Santé Publique du Québec, Sainte-Anne-de-Bellevue, QC) using the Sherlock Microbial Identification System® (MIDI, Inc., Newark, Del.).

Total FA concentration was the sum of all identified peaks greater than 0.5 pA and an ECL between 8.000 and 24.200. Peak area of each isolated peak was transformed to concentration (nmol g^{-1} d.w. soil or tissue), and the contribution of each FA to the total FA concentration in a sample was expressed as mole fraction (% mol). FAs are described by the standard ω-nomenclature A:BωC (IUPAC-IUB, 1977).

2.4. Fatty acid signature biomarkers

Interpretation of the FA profiles was aided by the use of FA biomarkers, since certain compounds are known to be associated with specific groups of organisms (Federle, 1986; O'Leary and Wilkinson, 1988; Vestal and White, 1989; Cavigelli et al., 1995; Frostegård and Bååth, 1996; Zelles, 1997). In particular, straight monounsaturated fatty acids (MUFAs) 16:1ω7c, 18:1ω7 and 15:1ω5 have been used as biomarker FAs for Gram-negative bacteria, and the branched saturated i15:0, a15:0, i16:0 and i17:0 for Gram-positive bacteria. All of these FAs plus the saturated odd numbered 15:0 and 17:0 FAs have been used as general bacterial FAs biomarker. The FAs 18:2ω6c and 18:1ω9c were considered to be mainly of fungal origin and the 10Me-18:0 to be an actinomycete biomarker FA. We assumed that the long-chain polyunsaturated fatty acids (LC-PUFAs) 20:3ω6 and 20:4ω6 were mainly from protozoa and other soil fauna.

2.5. Statistical analyses

Repeated measures ANOVA were used to detect differences among subjects (bulk soil, gut soil, gut wall and body wall). Sphericity was evaluated by Mauchley's test and *P*-values were corrected according the Greenhouse/Geisser adjustment of degrees of freedom. Variables were log-transformed to meet ANOVA requirements when needed. All analyses were performed using the GLM procedure and a probability level of $\alpha = 0.01$ was selected for separation of means. FAs concentration data (nmol g^{-1} d.w.) were analysed separately for soil samples and for tissue samples due to they are in a soil or tissue d.w. basis, respectively. The regression analyses were performed on the whole set of data, and significance was evaluated at $\alpha = 0.001$ due to the high sample size. Multivariate repeated measures ANOVA were performed to test differences in the FA composition by FA subgroups (grouped according their nature, expressed as % mol) between bulk soil and gut soil, and between gut wall and body wall. Cluster analysis was carried out on the normalized data (mole fraction) of all the identified FAs of ECL shorter than 20C, supposedly of microbial origin. Distances were measured as Euclidean distances and Ward's method was selected to linkage. Discriminant function analysis was performed on the mole fraction data of the identified FAs considered to come from bacterial or fungal cells. All analyses were conducted using Statistica 6.0. Values are showed as mean ± standard error of the mean.

3. Results

3.1. Fatty acid concentration and composition in bulk soil and gut soil

The concentration of total FAs in gut soil (3845 ± 992 nmol g^{-1} d.w.) was one order of magnitude higher than bulk soil (592 ± 129 nmol g^{-1} d.w.; $P < 0.001$). In spite the existence of a significant positive relationship, the concentration of microbial-derived FAs already present in bulk soil explained only the 50% of the concentration of FAs present in gut soil (Fig. 1a). Several bacterial FAs showed similar concentration in the gut as in bulk soil (16:1ω5, 16:1ω7, 16:1ω9; Fig. 1a), while other bacterial and fungal FAs were markedly more abundant in gut soil than in bulk soil (18:1ω7, 18:1ω9, i15:0 and other FAs over the 1:1 slope in Fig. 1a), showing any process leading to fractionated enrichment of microbial material in the gut soil.

Several LC-FAs probably of plant origin (20:0, 22:0, 23:0, 21:1ω9) appeared in similar concentration in bulk soil and in gut soil (Fig. 1b), and the concentration of typical soil microfauna FAs (20:4ω6, 20:5ω3) was much greater in gut soil than bulk soil. However, there was no significant relationship between LC-FAs in bulk soil and gut soil (Fig. 1b).

In the bulk soil, the FA with the greatest % mol concentration was the fungal biomarker 18:2ω6c, followed by the saturated 16:0. Bacterial-derived MUFAs, especially Gram-positive biomarkers 16:1ω7 and 18:1ω7, were also well represented (Table 1). The major FA compounds in gut soil were the eukaryote PUFA 20:4ω6, the fungal

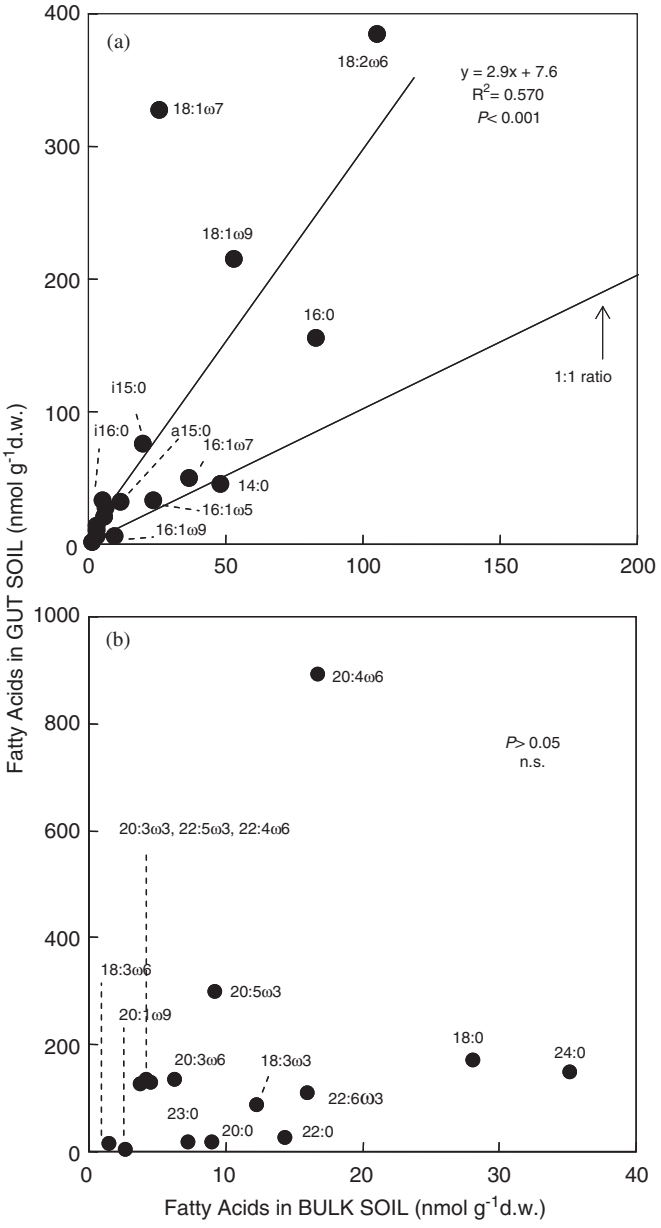


Fig. 1. Scatterplot of the concentration of fatty acids present in the bulk soil and in the gut soil of the earthworm *L. terrestris*. Fatty acids known to be of bacterial or fungal origin (a) are represented separately from long-chain FAs, probably derived from soil fauna or plant residues (b). Each point summarizes mean values ($n = 7$).

18:2ω6 and the bacterial monoenoic 18:1ω7 (Table 1). Several branched-chain FAs were also found.

Bulk soil showed greater mole fraction of identified SATFAs, MUFAs, short-chain PUFAs (SC-PUFAs) and branched-chain FAs than the gut soil (Table 2). Notably, the very long-chain SATFAs typical from plant residues present in bulk soil samples were not as prevalent (lower mole fraction) in gut soil. On the other hand, greater mole fractions of long-chain PUFAs were detected in gut soil than bulk soil (Table 2).

Table 1
Total fatty acid pattern in the bulk soil and in the gut soil, gut wall and body wall of the earthworm *L. terrestris*

Fatty acid[a]	Mole fraction of FAs (% mol)			
	Soil		Earthworm tissue	
	Bulk soil	Gut soil	Gut wall	Body wall
Straight-chain saturated FAs				
12:0	0.41±0.13	0.26±0.08	1.01±0.27	0.04±0.01
13:0	0.23±0.07	0.02±0.01	0.2±0.03	0.04±0
14:0	6.98±1.22	1.26±0.13	2.27±0.19	0.98±0.04
15:0	1±0.03	0.53±0.04	0.89±0.05	0.53±0.03
16:0	**13.32±0.38**	4.64±0.53	4.78±0.4	2.64±0.22
17:0	0.44±0.12	0.34±0.09	1.62±0.09	2.03±0.04
18:0	4.79±0.33	4.05±0.51	7.94±0.38	8.7±0.12
20:0	1.6±0.18	0.54±0.09	0.13±0.01	0.2±0.01
21:0	0.23±0.06	N.D.	0.02±0.01	0.01±0.01
22:0	2.5±0.24	0.88±0.19	0.04±0.01	0.06±0
23:0	0.92±0.25	0.72±0.47	0.04±0.04	0.08±0.02
24:0	4.86±0.86	4.82±1.2	0.98±0.42	0.24±0.03
Straight-chain monounsaturated				
14:1ω5	0.28±0.11	N.D.	0.01±0	N.D.
16:1ω5	4.04±0.31	1.07±0.19	0.9±0.09	0.44±0.05
16:1ω7c	5.89±0.33	1.46±0.19	1.31±0.19	0.49±0.02
16:1ω9	1.55±0.16	0.19±0.13	0.38±0.02	0.42±0.04
17:1ω7	N.D.	N.D.	N.D.	0.07±0.03
18:1ω7	4.35±0.31	**8.14±0.62**	**8.66±0.27**	**10.42±0.31**
18:1ω9c	**8.89±0.44**	6±0.55	6.38±0.69	6.64±0.14
20:1ω9	0.36±0.09	0.12±0.08	0.3±0.03	0.56±0.05
22:1ω9	0.11±0.11	N.D.	N.D.	N.D.
Straight-chain polyunsaturated				
18:2ω6c	**17.22±0.86**	**10.2±0.94**	**12.3±1.33**	5.77±0.56
18:2ω6t	N.D.	N.D.	N.D.	0.1±0.02
18:3ω3	2.25±0.28	2.4±0.21	2.74±0.31	2.9±0.34
18:3ω6	0.2±0.05	0.27±0.1	0.28±0.04	0.09±0.01
20:3ω3	0.78±0.13	2.73±0.28	2.49±0.21	1.76±0.08
20:3ω6	0.94±0.16	3.09±0.38	4±0.28	4.61±0.21
20:4ω6	2.89±0.27	**20.54±1.93**	**15.92±0.71**	**15.86±0.96**
20:5ω3	1.69±0.36	7.16±1.09	**9.56±1.1**	**21.46±1**
22:4ω6	0.45±0.13	3.01±0.22	1.55±0.12	1.88±0.11
22:5ω3	0.67±0.67	4.6±2.84	2.23±0.51	2.66±0.17
22:6ω3	1.96±0.55	2.92±0.42	1.3±0.21	1.2±0.07
Branched-chain FAs				
a15:0	1.9±0.1	0.94±0.13	1.42±0.11	0.56±0.03
i15:0	3.22±0.1	1.97±0.16	3.4±0.21	2.23±0.05
i16:0	0.9±0.05	0.83±0.06	1.37±0.04	1.19±0.02
cyc17:0	0.45±0.11	0.11±0.06	0.46±0.1	0.18±0.02
i17:0	1.12±0.08	0.72±0.06	0.87±0.06	0.81±0.03
a18:0	N.D.	0.1±0.06	1.22±0.32	0.01±0.01
cyc19:0	N.D.	N.D.	N.D.	0.05±0.02
i19:1	N.D.	0.03±0.02	0.18±0.03	0.22±0.02

The three major fatty acids in each column are marked in bold. Mean±SEM , $n = 7$.
N.D., undetected or below 0.05% mol in all replicates.
[a]The FAs 10:0, 11:0, 24:1ω9 and 2OH-12:0 were detected bellow 0.05% only in some replicates.

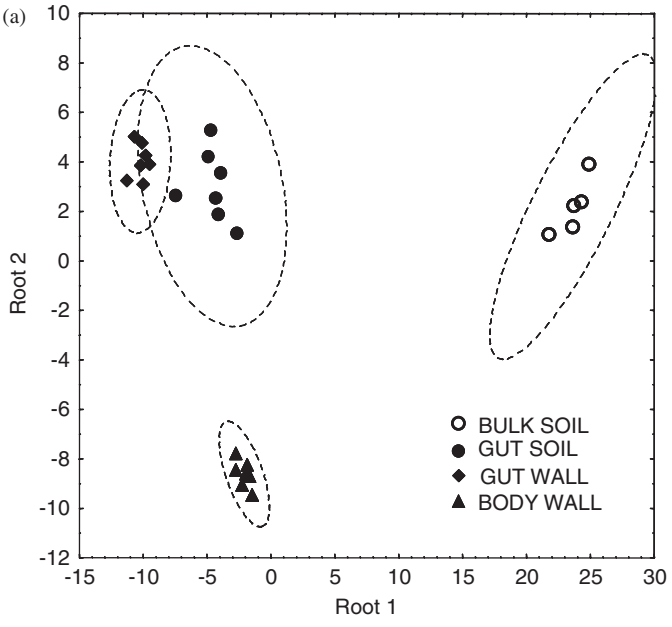
Considering the mole fraction of all the FAs grouped by their nature, bulk soil samples showed a FA composition significantly different than gut soil (RM-MANOVA Wilks

Table 2
Mole fraction of the identified fatty acids extracted from the bulk soil, gut soil, gut wall and body wall of the earthworm *L. terrestris* grouped according their structure (SATFAs, straight-chain saturated fatty acids; MUFAs, monounsaturated FAs; SC-PUFAs, short-chain polyunsaturated FAs, less than C20; LC-PUFA, long-chain PUFA, C20 or greater chain length; Branched, branched-chain saturated FAs)

		Mole fraction (% mol) of FAs in soil or earthworm tissue				
		ΣSATFAs	ΣMUFAs	ΣSC-PUFAs	ΣLC-PUFAs	ΣBranched
Soil	Bulk soil	37.3±1.2 a	25.5±0.7 a	19.7±1 a	9.4±1.4 c	2.5±0.1 b
	Gut soil	18.1±1.5 bc	17±1.3 b	12.9±1.1 b	44±3.1 a	1.8±0.1 c
Earthworm tissue	Gut wall	20±0.7 b	17.9±1.1 b	15.3±1.5 b	37±2.5 b	4.1±0.3 a
	Body wall	15.5±0.3 c	19.1±0.5 b	8.9±0.5 c	49.4±1.2 a	2.4±0.1 bc

Means in the same column followed by different letters are significantly different (RM-ANOVA, Tukey HSD Test, $P<0.001$). Mean±SEM., $n=7$.

(a)



(b)

	Standardized Canonical Function Coefficients			Correlation Coefficients between Discriminant Variables and Functions		
Fatty acid	Root 1	Root 2	Root 3	Root 1	Root 2	Root 3
13:0	2.87	-1.37	0.07	0.039	0.104	-0.241
a15:0	-3.55	2.15	0.54	0.089	0.256	-0.214
i16:0	-0.67	0.56	-1.80	-0.083	-0.069	-0.433
16:1ω5	3.54	-0.71	-0.12	0.243	0.220	-0.075
16:1ω7	1.18	1.17	0.33	0.305	0.294	-0.112
16:1ω9	0.31	-1.64	-0.61	0.157	0.036	-0.162
i17:0	0.71	-0.70	0.86	0.064	0.030	-0.139
cyc 17:0	-0.25	-0.73	-1.57	0.020	0.069	-0.187
18:1ω7	0.53	-0.38	0.82	-0.131	-0.213	0.004
18:1ω9	0.70	-2.37	-0.58	0.063	0.004	-0.064
18:2ω6	-1.81	1.91	-0.61	0.080	0.222	-0.106
18:3ω6	-0.74	0.84	0.83	-0.012	0.092	0.006
Eigenvalue	168.7	32.7	16.5			
Cum. Prop. %	77.4	92.4	100.0			
Funct. *P* value	< 0.0001	< 0.0001	< 0.0001			

Fig. 2. Results of discriminant function analysis (DFA) performed on identified FAs of bacterial and fungal origin from samples of bulk soil and gut soil, gut wall and body wall of the earthworm *L. terrestris*. In the Root 1 and Root 2 biplot (a) the dotted lines indicate the confidence ellipses at $\alpha = 0.05$. DFA discriminated 100% of samples using 3 functions and the 12 FAs in the table (b).

$F_{(1,6)} = 12$; adjusted (G–G) $P = 0.013$). Discriminant function analysis performed on the identified microbial-derived FAs also clearly differentiated bulk soil and gut soil samples in 100% of cases at $P<0.0001$ (Fig. 2a; global Wilks Lambda = 0.00001; $F_{(36,33)} = 44.5$;) with 12 selected more informative FAs included in the discriminant

function (Fig. 2b), providing evidence of distinct fatty acid compositions in bulk soil and gut soil.

3.2. Fatty acid concentration and composition in earthworm tissues

The concentration of total FAs in the gut wall ($87043 \pm 12893\,\mu mol\,g^{-1}$ d.w. tissue) was two-fold greater than in the body wall ($34778 \pm 2803\,\mu mol\,g^{-1}$ d.w. tissue; $P<0.01$). The major compounds in both tissues (Table 1) were the PUFAs 20:4ω6, 20:5ω3 and 18:2ω6, the MUFAs 18:1ω7 and 18:1ω9c, and the saturated 18:0, although the % mol of each compound was not uniform in the gut wall and body wall (Table 1). Many bacterial and fungal-derived FAs were found in earthworm tissues (Table 1), accounting for about 30–40% mol, where the absorptive gut wall contained a greater mole fraction of Gram-positive and fungal-derived FAs than the body wall ($P<0.001$). The discriminant function analysis performed on the microbial-derived FAs found in the gut wall and in the body wall clearly identified the two groups of samples in 100% of cases at $P<0.0001$ (Fig. 2a; Wilks Lambda = 0.00001; $F_{(36,33)} = 44.5$).

The body wall showed lower richness in SATFAs, branched FAs and SC-PUFAs than the gut wall (Table 2). Multivariate RM-ANOVA performed on the FAs grouped by their nature identified the gut wall samples significantly different from the body wall samples (Wilks $F_{(1,6)} = 15.9$; adjusted (G–G) $P = 0.0071$). On the other hand, the body wall contained greater levels of LC-PUFA than the gut wall. The concentration in the gut wall of FAs derived from the essential 18:2ω6 (the ω6 family of PUFA) was greater than ω3 PUFAs (derived from 18:3ω3), corresponding to a greater concentration of the ω6 precursor (18:2ω6) than the ω3 precursor (18:3ω3) in the gut soil (Table 1).

3.3. Trophic transfer and allocation of fatty acids from soil to earthworm tissue

We found bacterial and fungal FAs, which is supposed that earthworms cannot synthesize, in gut wall and body wall tissues (Table 1), suggesting trophic transfer and incorporation of intact soil microbial FAs into the EL-FAs fraction of *L. terrestris*. In order to confirm the possible direct uptake of microbial fatty acids into the earthworm tissues, we analysed the fatty acid profile in the neutral lipid fraction, which are the storage lipids in eukaryotes. In the earthworm gut wall at the midgut and the hindgut, we found the same FAs of microbial origin in the neutral lipid fraction of these tissues, confirming our total FAs results (Table 3). As expected, the profile of the NLFAs in the gut wall of *L. terrestris* was comprised mainly of animal FAs (20:3ω6, 20:4ω6, 18:0) accounting for 45 ± 1.9% mol. However, the joint contribution of identified bacterial and fungal-derived FAs was quite relevant, accounting for 31 ± 0.3% mol. The major FAs of bacterial origin found were 18:1ω7 (6.1 ± 0.33% mol), 16:1ω9 (3.1 ± 0.24% mol) and a15:0 (3.1 ± 0.69% mol). The joint contribution of branched FAs was about 14% mol, bacterial MUFAs was 10.8 ± 0.1% mol, and microbial-derived saturated FAs (15:0 and 17:0) was 6.2 ± 0.3% mol.

Table 3
Concentration of neutral lipid fatty acids in the gut wall tissue at the midgut and hindgut of the earthworm L. terrestris. Several microbial FAs were found in this fraction of storage lipids in the earthworm tissues.

Fatty acid (NLFA)	% mol in the earthworm gut wall
Straight-chain saturated FAs	
13:0	0.1 ± 0.002
14:0	0.7 ± 0.17
15:0	3 ± 0.45
16:0	4 ± 0.81
17:0	3 ± 0.22
18:0	14 ± 0.97
20:0	0.9 ± 0.08
22:0	1 ± 0.11
24:0	0.7 ± 0.05
Branched-chain FAs	
a15:0	3 ± 0.69
i15:0	2 ± 0.36
i16:0	2 ± 0.20
i17:0	2 ± 0.22
cyc17:0	1 ± 0.19
a17:1	4 ± 0.79
Straight-chain monounsaturated	
14:1ω5c	0.4 ± 0.04
16:1ω7c	1 ± 0.13
16:1ω9c	3 ± 0.24
17:1ω7c	0.4 ± 0.06
18:1ω7c	6 ± 0.33
18:1ω9c	7 ± 0.92
Straight-chain polyunsaturated	
18:2ω6c	4 ± 0.75
18:3ω6c	2 ± 0.23
20:2	4 ± 0.63
20:5ω3	2 ± 0.30
20:3ω3	0.3 ± 0.02
20:3ω6	21 ± 2.03
20:4ω6c	11 ± 0.96

Only FAs found at concentration greater than 0.05% mol in all replicates are shown. Mean $\pm$ SEM , $n = 5$.

Cluster analysis (Fig. 3) of the whole profile of identified microbial-derived FAs plainly separated the FA profile in bulk soil samples far apart from the gut soil samples, indicating a unique microbial FA profile in the gut soil. Besides, microbial-derived FA profiles of earthworm tissues appeared more closely related to the microbial community of the gut soil than to that in the bulk soil (Fig. 3). These results suggest that the microbial FAs incorporated by *L. terrestris* were more likely transferred from microorganisms present in the gut soil than from those in the bulk soil.

The concentration of microbial-derived FAs allocated in the gut wall showed a significant and strong linear

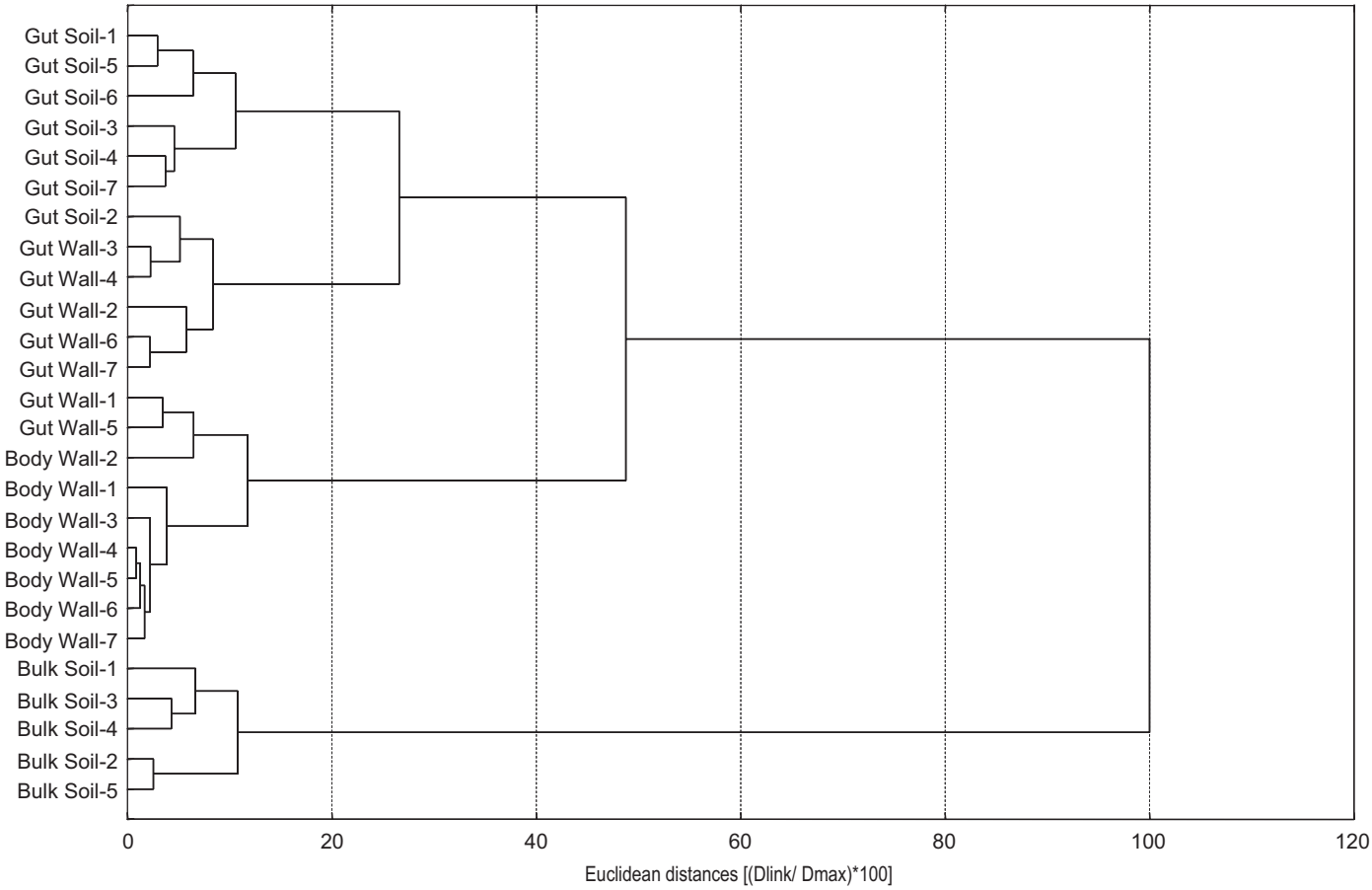


Fig. 3. Cluster analysis of the fatty acid profiles of bacterial and fungal origin extracted from samples of bulk soil, gut soil, gut wall and body wall of the earthworm *L. terrestris*.

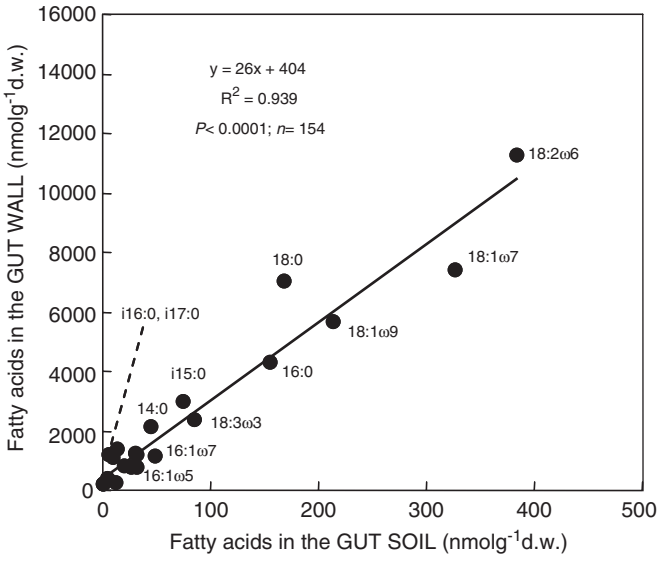


Fig. 4. Relationship between the concentration of fatty acids known to be of bacterial or fungal origin present in the gut soil and in the gut wall of *L. terrestris*. Each point summarizes the mean value of all cases ($n = 7$).

relationship to those present in the gut soil (Fig. 4a). That relationship was consistent, and even stronger, when all the cases were analysed separately, but showing different slopes among each individual culture bin (Fig. 4b). Furthermore, when considering microbial-derived FAs mole fractions, the relationship was also significant (figure not shown; $R^2 = 0.879$; $P < 0.0001$) with a slope = 1.1, suggesting that the entire suite of microbial FAs in the gut soil were readily absorbed, without bias, from the gut soil and stored in the gut wall of earthworms, although this possibility should be further investigated.

The transfer of microbial-derived and other FAs present in the gut wall to the body wall occurred at equimolar rate (Fig. 5, note the slope). This relationship was equally strong when only microbial-derived FAs were plotted (slope = 1.1; $R^2 = 0.931$; $P < 0.0001$), showing as well an equimolar transfer ratio of those FAs assimilated from the gut soil. The earthworm body wall, mainly comprised by muscular layers, showed a mole fraction of EL-FAs that resembled at great extent the absorptive tissue of the gut wall. But two marked exceptions were observed, where the body wall showed increased levels (just twice) of 20:5ω3, which have a recognized physiological role in animals, while depleted the fungal biomarker 18:2ω6. When this two FAs were excluded from the regression analysis, the relationship between the FAs in the body wall and gut wall was even stronger ($R^2 = 0.956$, $P < 0.0001$,

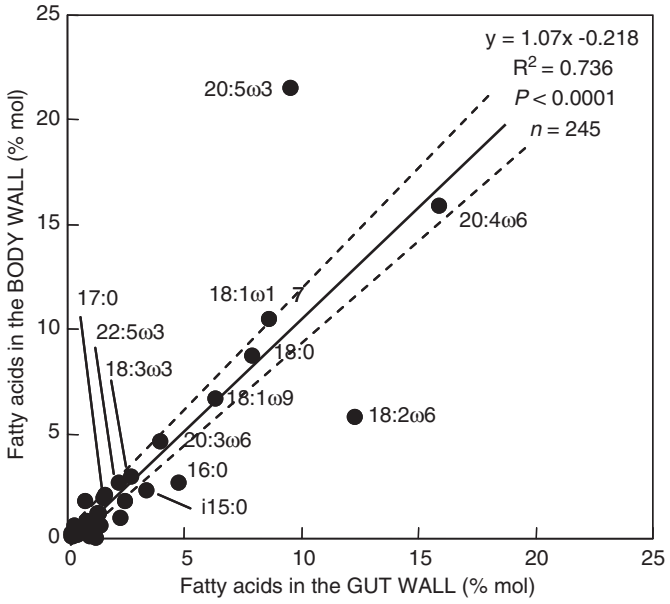


Fig. 5. Relationship between the mole fraction of fatty acids present in the gut wall and in the body wall of the earthworm *L. terrestris*. Dotted lines indicate 95% confidence level. Fatty acids are showed as mean values ($n = 7$).

slope = 1.02) supporting an equimolar allocation of the remaining FAs in gut wall and body wall.

4. Discussion

4.1. Fatty acid pattern and concentration in the bulk soil and the gut soil

Multivariate analyses, namely MANOVA, discriminant function analysis and cluster analysis indicated that the patterns of microbial derived FAs in the gut soil of *L. terrestris* was distinct from those found in the bulk soil. The existence of a microbial community in the gut that differ from that in the bulk soil has been probed for several soil organisms and it has also been suggested for earthworms by studies using other tools such as FISH and rRNA probes (Fischer et al., 1995; Schonholzer et al., 2002, 2003, 2004; Singleton et al., 2003, 2004). In addition to different composition, other studies have reported greater microbial numbers in the gut of earthworms than in bulk soil, also consistent with our results, using direct counts and culture-dependent methods (Parle, 1963; Márialigeti, 1979; Kristufek et al., 1992; Pedersen and Hendriksen, 1993; Karsten and Drake, 1995; Schonholzer et al., 1999).

The earthworm *L. terrestris* is known to selectively ingest plant debris and microbe-rich soil particles, which could partially explain why the FA concentrations were greater in the gut soil than the bulk soil. However, the earthworms we used came from culture bins with thoroughly homogenized soil and no added food, so there was little opportunity for the earthworms to engage in selective feeding behaviour. Yet, researchers who wish to use FA biomarkers to study trophic transfer between microorganisms and earthworms from the field should not overlook this possibility. Selective feeding may also affect FA digestion in the earthworm gut, and warrants further investigation.

4.2. Fatty acid composition in earthworm tissues

The nature and relative contribution (% mol) of major FAs detected in *L. terrestris* tissues concur with previous studies that found some long-chain PUFA, the MUFA 18:ω7 and 18:1ω9, and 18:0 to be among the major FAs in this earthworm species (Albro et al., 1992, 1993). The long chain-PUFAs 20:5ω3 and 20:4ω6 were previously found the two major components of total FAMEs and PLFA profiles in the lumbricid earthworms *Lumbricus rubellus*, *Aporrectodea caliginosa* and *Eisenia nordenskioldi* (Hansen and Czochanska, 1975; Petersen and Holmstrup, 2000) and in the Japanese anecic earthworm *Pheretima hilgendorfi* (Enami et al., 2001). However, the major PLFA in mitochondrial membranes of *L. terrestris* appeared to be the monounsaturated 20:1 and 18:1 (Crockett et al., 2001). Some of these studies have reported also the presence of some branched and other bacterial derived FAs in the whole fat body and in the PLFA of earthworms (Albro et al., 1992; Petersen and Holmstrup, 2000; Crockett et al., 2001).

The concentration of long-chain PUFAs found in earthworm tissues (about 40% mol) was greater than those usually found in other members of soil fauna. The concentration of long-chain PUFA reported for collembola and plant parasitic, entomopathogenic, fungivorous or bacterivorous nematodes ranged from less than 1% to 8% mol (Patel and Wright, 1997b; Chen et al., 2001; Ruess et al., 2002; Haubert et al., 2004; Chamberlain et al., 2004). The major FAs reported in nematodes are usually C18 FAs, especially 18:1ωù6, 18:2ω6 and 18:0 (Hutzell and Krusberg, 1982; Patel and Wright, 1997a,b; Fitters et al., 1999; Chen et al., 2001; Ruess et al., 2002, 2004). Prevalence of C18 and C16 is common also in Collembola (Haubert et al., 2004; Ruess et al., 2004, 2005; Chamberlain et al., 2004). In contrast to these reports, we found the C20 FAs were a major component of FAs in *L. terrestris* tissues. However, Chamberlain and Black (2005) also detected unusually high concentrations (up to 30% of total FAs) of C20 PUFA in five species of Collembola raised on yeast diets, and as much as 20% mol of the PUFA 20:4ω6 was also found in a bacterivorous nematode species (Ruess et al., 2004). Clearly, more information about the physiological role of these compounds in soil fauna is needed.

We observed a significantly greater concentration of FAs in the gut wall tissue than in the body wall. The cells in the gut wall of Oligochaeta have long been recognized for their importance in lipid storage (Stephenson, 1930). Unusual granular fatty reserves into the absorptive cells of the intestine of *L. terrestris,* comprised mainly (up to 87%) by triglycerides, were described in an early paper by Willem

and Minne (1899, cited by Stephenson, 1930), suggesting the main fraction of the fat body in earthworm gut wall could be composed of storage neutral lipids, while FAs in the body wall would be mostly membrane lipids. Differential allocation between NLFA and PLFA in earthworm tissues (Albro et al., 1992), and diverse turnover rates of lipids in body tissues as reported by Chamberlain et al. (2004) in collembola, could likely explain the differences in mole fraction of some FAs in gut wall and body wall (Fig. 3). The FAs that appeared in lower % mol in the gut wall than in the body wall (compounds under the slope = 1, Fig. 3) could belong to storage NLFA, whereas FAs that appeared enriched in the body wall may be constituents of membrane PLFA. According to previous findings indicating that NLFAs better reflect changes in the diet than polar lipids (Chen et al., 2001; Haubert et al., 2004), and to the present evidence of FA fractionation among earthworm tissues, we propose to extract the neutral lipids from the gut wall and the body wall separately, when possible. The gut wall would likely be the most interesting of these two tissues for tracing trophic transfer of FAs, especially in short-term studies.

4.3. Incorporation of dietary fatty acids to the earthworm tissues

Other studies of PLFA and whole-cell FAME composition in earthworms have extracted FAs from the entire earthworm, including the gut soil (McLaughlin, 1971; Hansen and Czochanska, 1975; Petersen and Holmstrup, 2000; Enami et al., 2001), or extracted FAs from earthworm tissues after starving the animal for 24 h and washing to remove gut soil (Albro et al., 1992). But, as far as we know, this is the first study of EL-FAs considering all the compartments involved in the trophic transfer of FAs, i.e. the diet (bulk soil), the gut soil with its associate microflora, the absorptive tissue (gut wall) and the main biomass, namely the muscular layers of the body wall. The presence of unmodified dietary lipids, such as plant lipids or bacterial and fungal derived FAs, in the body tissue of terrestrial invertebrates has been reported in hymenoptera (Stanley-Samuelson et al., 1988), collembola (Chamberlain et al., 2004; Haubert et al., 2004; Ruess et al., 2004) and nematodes (Chen et al., 2001; Ruess et al., 2002). It had been already suggested for earthworms (Albro et al., 1992; Petersen and Holmstrup, 2000) but not probed. Results from this study provide compelling evidence of trophic transfer of dietary lipids from microbes to earthworms. Further studies of dietary routing of FAs in different earthworm species as affected by dietary variables and environmental conditions will provide valuable information for tracing the complex trophic relationships involving microbes and earthworms, and for understanding the function of the gut microbiota in this group of soil fauna.

The patterns of bacterial and fungal FAs in the earthworm body wall were consistent with those in the gut wall, although their concentrations were lower in the muscular tissue than in the gut wall. Bacterial and fungal FA profiles in the earthworm tissue resembled those in the gut soil more than those in the bulk soil. Other authors have found that the FAME profile of the body tissue of *L. terrestris* was not directly related to the FAME composition of the supplied food (Albro et al., 1992), while bacterial derived FAs not present in the diet appeared in nematode and collembolan tissues (Ruess et al., 2002; Chamberlain et al., 2004; Haubert et al., 2004). A possible explanation, based on our findings, is that gut microorganisms supplied a wider range of FAs to their host than the pure diet did. It has been suggested that gut microbiota is also required to explain the amino acid composition of earthworms, since earthworms ingest almost 20 times less methionine and tryptophan than they require for their nutrition; therefore, these compounds must be synthesized into the gut (Pokarzhevskii et al., 1997). Furthermore, these findings suggest that it may be erroneous to assume that some FAs are synthesized by soil fauna when FAs not appearing in the diet are found in animal tissues, because gut microbiota could provide those FAs to their hosts.

The earthworm gut wall appears to store assimilated FAs in the form of neutral lipids. Transfer of assimilated bacterial and fungal FAs from the gut wall to the muscular layers in the body wall occurs, but not at the same rate for all assimilated FAs, possibly due to their preferential allocation as neutral lipids or as polar lipids. Finally, the body wall of *L. terrestris* appeared depleted in bacterial FAs regarding to the gut wall due to higher concentration of long-chain PUFA in the muscular tissue, mainly eicosapentaenoic acid (EPA, 20:5ω3), possibly a main compound in the PLFA pool.

Linoleate (18:2ω6) appearing in earthworm's tissues is a known fungal biomarker, however the presence of this FA in earthworm tissues cannot be used to demonstrate that earthworms feed on fungi because this FA is abundant in the tissues of soil microfauna. We cannot overlook the possibility that linoleate was assimilated from ingested nematodes or other soil micrometazoa in the earthworm gut. In addition, it has been shown that linoleate, long considered an essential FA, can be biosynthesized by several insects (Stanley-Samuelson et al., 1988; Canavoso et al., 2001). It is possible that linoleate may be biosynthesized by earthworms, although we are not aware of any reports that confirm or reject this possibility. More study is needed on this topic.

Our results indicate that FAs assimilated by *L. terrestris* come mostly from the gut microbiota rather than those in the bulk soil. This has important implications for the use of FA profiles to understand trophic transfers in soil food webs. Detritivorous soil organisms like earthworms may derive lipids from their gut microbiota, suggesting that the FA pattern in their tissues would be similar to the FA pattern of gut microbes. However, we expect that predatory (fungivorous or bacterivorous) soil organisms would have an FA pattern similar to that of the ingested material, based on the "you are what you eat" principle.

Further study is needed to determine if this trend occurs for other organisms in soil food webs.

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