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Changes in the fatty acid profiles through the digestive tract of the earthworm *Lumbricus terrestris* L.

Luis Sampedro [a,b,*], Joann K. Whalen [b]

[a] *Department of Natural Resource Sciences, Macdonald Campus of McGill University, 21.111 Lakeshore Road, Ste-Anne-de-Bellevue, Quebec, Canada H9X 3V9*

[b] *Departamento de Ecoloxía e Bioloxía Animal, Universidade de Vigo, E-36310 Vigo, Spain*

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Abstract

The gut of many soil arthropods contains a complex and mutualistic microbial community that usually assists the host with digestion. The same is probably true for earthworms, but the nature and function of the microbiota inhabiting their gut are virtually unknown. In this paper, we studied the microbial community in the gut content of the earthworm *Lumbricus terrestris* L. and in the bulk soil by assessing their fatty acid (FA) profiles. Our results indicated that the total FA concentration in the earthworm gut was about two orders of magnitude greater than in bulk soil, with higher concentration of bacteria (up to 500-fold), fungal and metazoan-derived FAs. Several FAs appearing in the gut were not present in bulk soil. PCA analysis revealed that the microbial community in the gut was different from that in the bulk soil, and that significant changes occurred between midgut, hindgut and proctodeum. Cluster analysis of bacterial and fungal-derived FA profiles grouped the bulk soil samples apart from the gut samples, where the hindgut profiles were more closely related to those from the proctodeum than those from the midgut. We showed important changes in the FA concentration and composition occurring at very small spatial scales inside the gut of the earthworm *L. terrestris*. These results have implications for understanding earthworm digestion, and they suggest that the microbial community in the earthworm gut is not a casual combination of microorganisms already present in the soil. Further study is needed to determine how these gut microbial communities are involved in earthworm digestion processes.

Keywords: Earthworm; Fatty acid; Gut microbiota; Digestive mutualisms

1. Introduction

The gut of many soil arthropods contains complex microbial communities that usually assist the host with digestion. These microbial–animal relationships range from commensalisms to species-specific mutualisms. Earthworms are also believed to have a mutualistic relationship with soil microorganisms passing through their digestive tract, but the nature and function of the microbiota inhabiting their gut are virtually unknown. It is hypothesized that mucus and water secretion from the earthworm gut wall into the digestive tract causes dormant, ingested microbiota to be activated, increasing microbial activity and enzyme production and hence facilitating digestion (Lavelle and Spain, 2001). This mucus-mediated, facultative mutualistic digestion was first proposed for tropical endogeic geophagous earthworms (Barois, 1992; Trigo and Lavelle, 1993). Anecic earthworms are thought to facilitate the digestion by mixing soil and plant residues to stimulate soil

* Corresponding author at: Departamento de Ecoloxía, Centro de Investigacións Ambientais de Lourizán, Apartado 127, E-36080 Pontevedra, Galicia, Spain. Tel.: +34 986 856078; fax: +34 986 856420.
E-mail address: lsampe@uvigo.es (L. Sampedro).

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microbial activity, effectively creating an ''external rumen'' before ingesting the partially decomposed materials (Lavelle and Spain, 2001). After organic substrates are consumed, earthworms also could release major enzymes necessary for decomposition into the gut, leading to ''direct digestion'' by endogenous enzymes.

Since Parle (1963) first reported the microbiology in the earthworm gut, researchers have attempted to study earthworm gut microbes using direct culture methods (e.g. Krištufek et al., 1992; Karsten and Drake, 1995), electron microscopy (Jolly et al., 1993), epifluorescence direct counts of DAPI or acridine orange stained prokaryotes (Krištufek et al., 1995; Wolter and Scheu, 1999) and more recently by fluorescent in situ hybridization with rRNA-targeted probes (FISH) specific for major bacteria phyla (Fischer et al., 1995; Schonholzer et al., 2002). Microbial numbers in the earthworm gut are higher than those in surrounding soil and denitrification, but not methane emission, occurs in the gut of *Aporrectodea caliginosa* and *Lumbricus rubellus* (Karsten and Drake, 1997). In addition, gene clone libraries of bacteria tightly associated with the gut wall were different from those in casts and in the gut content (Singleton et al., 2003, 2004). These findings suggest that the earthworm gut is favourable for the growth and activity of certain bacterial species, but whether this relationship is obligate or not, and its relevance in the energy uptake of the earthworm remains to be determined.

Fatty acid (FA) analysis is a common culture-independent tool for the study of microbial communities in sediments and soils. Depending on the procedure for extraction and the nature of the FA analyzed, it can provide information about the microbial biomass, microbial community composition, and physiological status (Haack et al., 1994; White and Ringelberg, 1998; Zelles, 1999; Schutter and Dick, 2000). The ester-linked fatty acid (EL-FA) fraction of environmental samples includes both polar lipids (membrane lipids related to biomass) and neutral lipids (storage lipids in eukaryotes) (Zelles, 1999). The EL-FA fraction has been successfully used to characterize shifts in the microbial community of soils and sediments (e.g. Schutter and Dick, 2000), but we are not aware of other published studies to examine microbial communities in the earthworm gut using FA profiles. In this study, we analyzed the FA profiles to gain insight into changes in the microbial community occurring along the digestive tract of the anecic temperate earthworm *Lumbricus terrestris* L.

2. Materials and methods

2.1. Earthworm collection and experimental design

Adult *L. terrestris* L. were collected by formalin extraction (0.25% formaldehyde) from a mixed grass–legume hayfield (loamy, mixed, typic endoaquent, pH 6.0 and 28 g C kg^{-1}), quickly washed in tap water and maintained in a culture bin at 18 °C in dark for 10 days with the original soil, thoroughly homogenized by sieving through 2 mm mesh, without added food to diminish interferences from selective feeding. Each earthworm was then cooled on ice for 5 min, which anesthetized them and prevented evacuation of the gut material, then euthanized in 50% ethanol solution, rinsed with sterile water, and immediately aseptically dissected on ice. The intestinal tract behind the gizzard was divided into midgut (closest to the mouth), hindgut and proctodeum (furthest from the mouth), and the gut content was carefully collected with dissecting needles under stereomicroscope (Stephenson, 1930; Harrison and Gardiner, 1992; Jamieson, 1992). This was quite time-consuming, so we included only 15 specimens dissected within a 48 h period to minimize the variation between individuals. Material from each gut section of five specimens was pooled to obtain at least 200 mg dry weight (dw) per replicate (there were three replicate samples from the midgut, hindgut and proctodeum). Samples of the gut content and samples of homogenised bulk soil ($n = 3$) from the culture bin (each about 2 g dw) were frozen, freeze dried and stored frozen until lipid extraction.

2.2. Lipid extraction and analysis

Total lipids were extracted with methanol and chloroform (2:1 v/v) using an accelerated solvent extraction with the Dionex ASE 200™ Extractor (Dionex Corporation, Sunnyvale, CA, USA, http://www.dionex.com) according to Macnaughton et al. (1997). Accelerated solvent extraction employs a combination of increased temperature and pressure with common solvents to increase the efficiency of the extraction process. Freeze dried samples were weighed into steel extraction cells, individually heated to 80 °C under N_2 and brought to 8.28 MPa for 5 min, then subjected to static extraction for 15 min. Three static cycles were conducted on each sample. Fatty acids methyl esters (FAMEs) were prepared by a mild alkaline methanolysis of the total lipid extract according to the standard procedure proposed by White and Ringelberg (1998). This procedure is suitable only for

transesterification of ester-linked fatty acids, and it does not methylate free FAs nor destroy the cyclopropane rings (Kates, 1986; Grogan and Cronan, 1997).

2.3. Quantification and identification of FAMEs

FAs were analyzed in split mode (50:1) with a gas chromatograph (Hewlett Packard 6890, Agilent Technologies) equipped with a Simplicity Wax capillary column (cross-linked PEG, Supelco 2-4326), He 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, resulting in a conversion factor of 0.62 ng U^{-1} of peak area. Identification of peaks was based on comparison of retention times to known 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-Hexadecenoate Methyl Ester cat. #1208 and Matreya 10-Methyloctadecanoate Methyl Ester cat. #1763) containing FAMEs with chain length ranging from 8 to 24 carbon atoms, allowing us to identify 53 FAMEs. The correct identification and quantification of those peaks was confirmed by duplicate analyses of a range of samples in a certified laboratory (Laboratoire de Santé Publique du Quebec, Sainte-Anne-de-Bellevue, Quebec, Canada) using the Sherlock Microbial Identification System® (MIDI Inc., Newark, Delaware).

Peak area of each isolated peak was transformed to concentration (nmol g^{-1} dw soil), and total FA concentration was the sum of all peaks with an area greater than 0.5 U and a retention time between 8:0 and 24:1ω9. The contribution of each FA to the total FA concentration in a sample was expressed as mole fraction (relative richness, mol%).

2.4. FA nomenclature

FAs are described by standard IUPAC-IUB (1977) ω-nomenclature *A*:*B*ω*C*, where "*A*" indicates the total number of C atoms, "*B*" the number of unsaturated bonds and "ω*C*" indicates the number of C atoms between the aliphatic end of the molecule and the first unsaturated bond. *cis* and *trans* isomers are indicated by the suffixes *c* and *t*, respectively. The prefixes *i* and *a* indicate *iso* and *anteiso* methyl branching, respectively. Other notations are "cy" for cyclopropyl groups, *X*Me for mid-chain methyl groups and *X*OH for hydroxyl groups, where "*X*" refers to the number of carbons from the carboxyl end of the molecule.

2.5. FA signature biomarkers

Interpretation of the FA profiles was aided by the use of FA biomarkers, since certain FAs are known to be associated with specific groups of organisms (e.g. Federle, 1986; O'Leary and Wilkinson, 1988; Vestal and White, 1989; Cavigelli et al., 1995). In particular, straight monoenoic FAs 16:1ω7*c*, 18:1ω7 and 15:1ω5 have been used as biomarker FAs for Gram-negative bacteria, and the branched saturated *i*15:0, *a*15:0, *i*16:0 and *i*17:0 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 18:2ω6*c* and 18:1ω9*c* were considered to be mainly of fungal origin and the 10Me-18:0 to be an actinomycete biomarker FA. We assumed that long chain FAs 22:0, 24:0, 20:1ω6 and 22:1ω6 already present in soil were from plant residues and the eukaryote polyunsaturated FAs (PUFAs) 20:3ω6 and 20:4ω6 were mainly from the soil fauna (e.g. Aitzetmuller, 1996; Albro et al., 1992; Griffiths et al., 1999).

2.6. Statistical analyses

The data matrix for multivariate analyses performed on FA profiles consisted of mol% of FAs with a concentration higher than 0.1 mol% present in all three replicates of at least one treatment (bulk soil, hindgut, midgut or proctodeum). The FAs that did not contribute to total variability, as determined in preliminary analyses, were excluded from the analysis to reduce the number of variables to less than the number of observations. Also FAs known to be present in earthworm tissues were discarded in order to avoid the interference of possible contamination of gut soil with FAs from earthworm tissues. PCA scores after Varimax normalized rotation were subsequently analyzed by ANOVA. Cluster analysis of FAs was performed on the normalized data (mol%) of all (known and unknown) isolated peaks after discarding palmitate (16:0) and stearate (18:0) because of they are ubiquitous in organisms, and the compounds with chain length $\geq$20C due to their uncertain origin (plant tissues, microfauna, protozoa or even earthworm tissue). Distances were measured as Euclidean distances and linkage was done using Ward's method. Data were

transformed to equalize variance and normality when needed, and differences between treatments were determined using the GLM procedure, followed by a Tukey's HSD test for separation of means. Significance was evaluated at $\alpha = 0.05$ for all tests. All analyses were conducted using Statistica 6.0. Values presented in the tables and figures are the mean ± standard error of the mean (S.E.M., $n = 3$).

3. Results

3.1. FA composition and concentration in bulk soil and the earthworm gut

The total FA concentration in the hindgut was as much as 300 times greater than bulk soil ($F_{(3,8)} = 12.6$, $P = 0.002$; Table 1) with intermediate concentrations in the midgut and the proctodeum. The FAs identified using the commercial standards accounted for 56–72 mol% of the FAs extracted (Table 1).

Nine FAs, mainly very long chain FAs (LC-FAs), accounted for about 50 mol% in the bulk soil. Two unidentified compounds (Unk1.389, 12.5 mol%; Unk1.315, 7.9 mol%) were the most abundant, followed by 24:0, 20:5ω3, and three unknown FAs (Unk0.597, Unk1.462 and Unk0.719; joint contribution 11.8 mol%). Several bacterial, fungal and protozoan biomarkers were identified in the bulk soil, however, the odd numbered saturated FAs 15:0 and 17:0, the branched FAs and the 10Me18:0 were not detected (Table 2). Two unidentified FAs (Unk1.076 and Unk1.145) were unique in the bulk soil, accounting for ca. 5 ± 2.3 mol%.

The polyenoic 20:4ω6 was the most abundant FA in all the samples from the earthworm gut (Table 2), accounting for about 15 mol%. In the midgut, 52 mol% was accounted by seven FAs, and the second major compound was Unk0.804 (9.5 mol%), followed by 18:1ω7, 18:2ω6 and 20:5ω3. Major FAs in the hindgut and proctodeum showed a similar trend, where 50% of the mole fraction was accounted by 20:4ω6, followed by 20:5ω3, 18:1ω7, Unk1.103 (about 6.5 mol%) and 18:0. We found 24 FAs common to the three gut sections that were absent from the bulk soil; these FAs accounted for about 15 mol% of the total FA concentration in the earthworm gut. They included bacterial FAs *i*15:0, *i*16:0, 17:0 and unidentified FAs belonging to the 15C and 16C series, several eukaryotic FAs such as 22:4ω6, 22:5ω3 and 22:6ω3 and other unidentified LC-FAs. The cy17:0, usually abundant in anaerobic eubacteria, was found only in midgut and hindgut. Besides, some rare FAs were present in specific parts of the gut: the Unk0.688 appeared only in the midgut (0.31 ± 0.02 mol%), two unknown LC-FA were found in the proctodeum, and the saturated 11:0 and other 10 unknown FAs with retention times between 14:0 and 15:0, supposedly of bacterial origin, appeared only in the hindgut (joint contribution of 0.45 ± 0.17 mol%).

The joint mole percentage of known polyunsaturated FAs (PUFA) in the gut was higher than in the bulk soil ($F_{(3,8)} = 5.21$, $P = 0.027$; Table 3), chiefly at the end of the alimentary tract. All the ω6 family of PUFAs coming from Δ^6 desaturation of linoleic acid (18:2ω6) and subsequent elongation and desaturation cycles (18:3ω6, 20:3ω6, 20:4ω6 and 22:4ω6) appeared enriched in the gut related to the bulk soil, especially the 20:4ω6. The same was observed for FAs coming from 18:3ω3 (α-linolenic), both for the direct elongation product (20:3ω3) as for the ω3 family coming from elongation–desaturation cycles (i.e. 20:5ω3, 22:5ω3 and 22:6ω3), particularly for 20:5ω3. The contribution of 20:1ω9 and 22:1ω9, products of the elongase pathway of the oleic acid 18:1ω9 was also greater in the gut than in bulk soil. On the other hand, the combined mole fraction of saturated FAs was greater in bulk soil than in the gut sections (Table 3).

Table 1
Total concentration of fatty acids (FA) and mole fraction of identified FAMEs in three sections of the gut content of the earthworm *L. terrestris* and in the bulk soil

	Total FA concentration (nmol g^{-1} soil dw)	Identified FAMEs (mol%)
Bulk soil	28 ± 6 b	56 ± 8 b
Gut content		
Midgut	6122 ± 1151 a	68 ± 2 ab
Hindgut	10156 ± 3162 a	72 ± 1 a
Proctodeum	4582 ± 1808 a	71 ± 1 a

Mean ± S.E.M.; $n = 3$; means in the same column followed by the same letters are not significantly different from each other (Tukey's HSD test, $P < 0.05$).

3.2. Biomarker FAs and FA profiles in bulk soil and earthworm gut sections

The bacterial community in bulk soil appeared to be composed mainly of Gram-negative bacteria (Fig. 1). The earthworm gut was enriched both in bacterial ($F_{(3,8)} = 8.2$, $P = 0.007$) and fungal biomarkers ($F_{(3,8)} = 4.2$, $P = 0.045$), compared to bulk soil and it appeared to be more favourable to Gram-positive bacteria than the bulk soil (Fig. 1). The earthworm gut contained more eukaryote biomarkers ($F_{(3,8)} = 4.7$, $P = 0.035$) than the bulk soil (Fig. 1). These FAs may

Table 2
Fatty acid pattern through the gut content of the earthworm *L. terrestris* and in the bulk soil

Fatty acid[a]	Retention time[b]	Relative richness in bulk soil or gut content (mol%)			
		Bulk soil	Midgut	Hindgut	Proctodeum
Straight chain saturated FAs					
10:0	0.300	0.81 ± 0.81	ND[c]	ND	ND
11:0	0.318	ND	ND	0.02 ± 0.01	ND
12:0	0.364	ND	0.1 ± 0.02	0.51 ± 0.02	0.08 ± 0.05
13:0	0.426	ND	0.06 ± 0.04	0.25 ± 0.02	0.02 ± 0.02
14:0	0.512	ND	0.62 ± 0.13	0.96 ± 0.04	0.68 ± 0.03
15:0	0.609	ND	0.58 ± 0.08	0.5 ± 0.01	0.29 ± 0.03
16:0	0.709	2.41 ± 0.29	1.92 ± 0.25	1.5 ± 0.01	1.21 ± 0.13
17:0	0.809	ND	1.11 ± 0.09	1.62 ± 0	1.24 ± 0.06
18:0	0.906	3.49 ± 0.19	4.29 ± 0.23	5.14 ± 0.02	4.02 ± 0.13
20:0	1.091	0.94 ± 0.94	0.03 ± 0.03	0.09 ± 0.04	0.03 ± 0.03
22:0	1.263	3.3 ± 0.92	ND	0.05 ± 0.02	0.09 ± 0.07
23:0	1.345	ND	ND	ND	0.02 ± 0.02
24:0	1.426	6.41 ± 0.81	1.49 ± 1.49	0.08 ± 0.04	0.2 ± 0.16
Branched-chain saturated FAs					
*i*15:0	0.562	ND	2.66 ± 0.49	2.58 ± 0.05	1.36 ± 0.21
*a*15:0	0.576	ND	1.3 ± 0.21	0.78 ± 0.01	0.43 ± 0.04
*i*16:0	0.661	ND	0.85 ± 0.15	1.34 ± 0.01	0.9 ± 0.12
*i*17:0	0.761	ND	0.64 ± 0.1	0.77 ± 0.02	0.63 ± 0.09
cy17:0	0.835	ND	0.12 ± 0.02	0.07 ± 0.04	ND
Hydroxy FAs					
3OH-18:0	1.205	ND	0.76 ± 0.05	0.98 ± 0.02	1.15 ± 0.04
Short chain monounsaturated					
15:1ω5	0.631	ND	0.04 ± 0.04	0.11 ± 0.05	0.04 ± 0.04
16:1ω9	0.727	ND	0.12 ± 0.06	0.05 ± 0.05	ND
16:1ω7*c*	0.733	ND	0.73 ± 0.07	0.54 ± 0.01	0.51 ± 0.02
16:1ω5	0.746	ND	0.82 ± 0.07	0.44 ± 0.02	0.5 ± 0.14
18:1ω9*c*	0.922	2.1 ± 1.05	3.87 ± 0.25	2.47 ± 0.08	2.85 ± 0.08
18:1ω7	0.930	3.22 ± 1.72	6.99 ± 0.57	8.01 ± 0.11	7.41 ± 0.31
Long chain monounsaturated					
20:1ω9	1.107	ND	0.04 ± 0.04	0.12 ± 0.06	0.14 ± 0.07
22:1ω9	1.279	ND	0.07 ± 0.07	0.11 ± 0.05	0.09 ± 0.09
Polyunsaturated					
18:2ω6*c*	0.965	2.44 ± 1.36	6.71 ± 0.15	5.08 ± 0.06	4.57 ± 0.18
18:2ω6*t*	0.971	ND	ND	ND	0.01 ± 0.01
18:3ω6	0.993	ND	0.15 ± 0	0.02 ± 0.02	0.02 ± 0.02
18:3ω3	1.025	0.99 ± 0.99	3.8 ± 0.09	3.56 ± 0.03	3.45 ± 0.01
20:2ω6	1.135	3.26 ± 0.71	0.07 ± 0.07	ND	0.46 ± 0.21
20:3ω6	1.148	1.98 ± 1.98	2.48 ± 0.16	3.79 ± 0.1	3.75 ± 0.18
20:3ω3	1.171	0.6 ± 0.6	3.06 ± 0.25	2.28 ± 0.03	2.1 ± 0.03
20:4ω6	1.190	3.89 ± 3.89	13.87 ± 1.12	15.28 ± 0.13	16.59 ± 0.47
20:5ω3	1.246	5.86 ± 1.98	6.49 ± 0.89	10.02 ± 0.06	11.89 ± 0.47
22:2ω6	1.319	3.08 ± 0.68	0.05 ± 0.05	0.04 ± 0.04	0.34 ± 0.08
22:4ω6	1.360	ND	0.96 ± 0.12	1.17 ± 0.03	1.62 ± 0.02
22:5ω3	1.414	ND	0.37 ± 0.2	0.96 ± 0.06	2.14 ± 0.7
22:6ω3	1.440	ND	0.32 ± 0.17	0.41 ± 0.03	0.22 ± 0.11

Mean ± S.E.M.; $n = 3$.

[a] Only identified peaks are shown. The following fatty acids were not detected in any sample of any treatment: *i*10:0; 10Me18:0; 2OH-10:0; 2OH-12:0; 3OH-12:0; 2OH-14:0; 3OH-14:0; 2OH-16:0; 14:1ω5; 17:1ω7; 18:1ω9*t*; 24:1ω9 and 21:0.

[b] Calculated as the relative retention time to the nonadecanoate retention time (19:0, IS).

[c] Below the detection limit of the instrument in all the replicates.

Table 3
Relative mole percentage of the identified FAs extracted from three sections of the gut content of the earthworm *L. terrestris* and from the bulk soil, grouped according their structure

	Relative richness in soil or gut content (mol%)					
	∑ SATFAs	∑ Branched	∑ Hydroxy	∑ SC-MUFAs	∑ LC-MUFAs	∑ PUFAs
Bulk soil	17 ± 2.9 a	ND[a] b	ND d	5 ± 2.7 b	ND	22 ± 9.4 b
Midgut	10 ± 1.6 ab	5.6 ± 0.9 a	0.8 ± 0.05 c	13 ± 1.0 a	0.1 ± 0.1	38 ± 1.9 ab
Hindgut	11 ± 0.2 ab	5.6 ± 0.1 a	1.0 ± 0.02 b	12 ± 0.3 ab	0.2 ± 0.1	43 ± 0.2 ab
Proctodeum	8 ± 0.2 b	3.3 ± 0.5 a	1.2 ± 0.04 a	11 ± 0.3 ab	0.2 ± 0.1	47 ± 0.4 a

Mean ± S.E.M.; $n = 3$; means in the same column followed by the same or no letters are not significantly different from each other (Tukey's HSD test, $P < 0.05$). SATFA: straight-chain saturated fatty acids; Branched: branched-chain saturated; SC-MUFA: short chain monounsaturated FAs, <20C chain length; LC-MUFA: long chain MUFA; PUFAs: polyunsaturated FAs.
[a] Below the detection limit of the instrument in all the replicates.

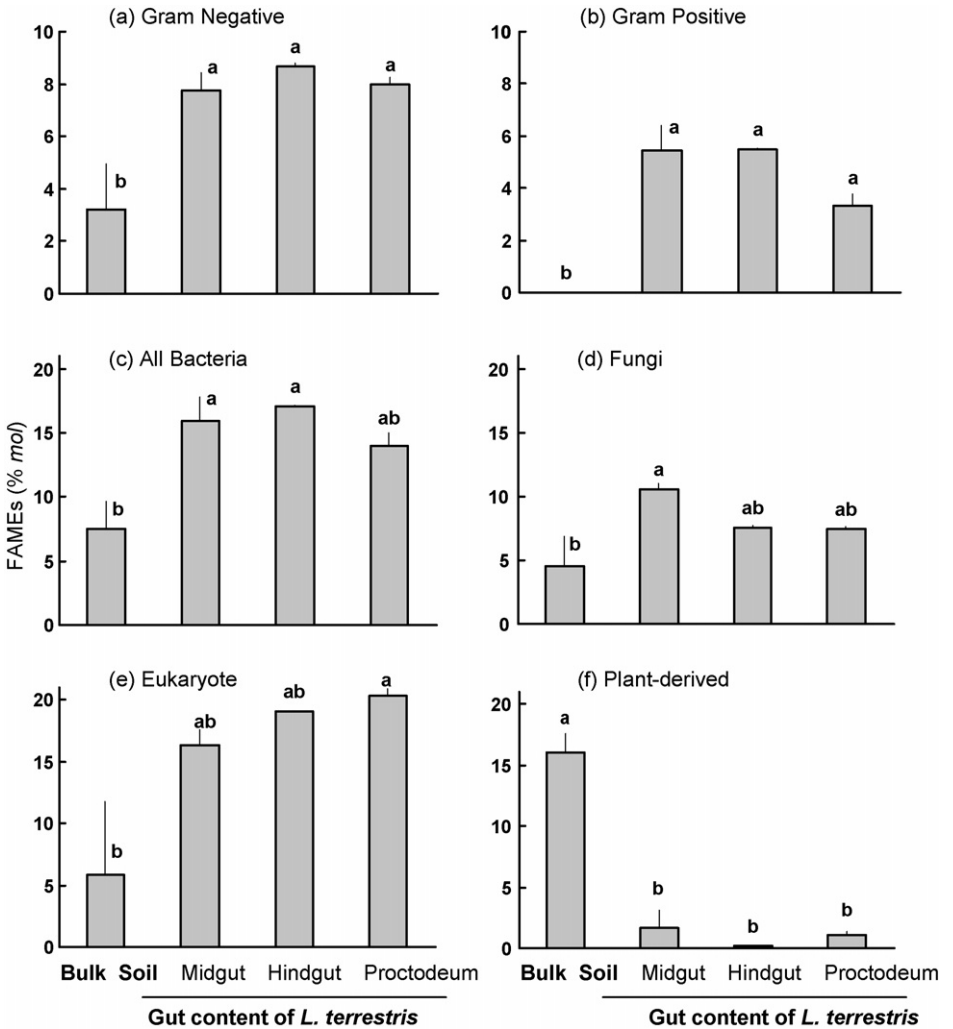

Fig. 1. Mole percentage of selected biomarker FAs through the gut content of *Lumbricus terrestris* and in bulk soil. (c) Includes Gram-negative, Gram-positive and general biomarker FAs for bacteria; (e) does not include fungi biomarkers. For more explanation see Section 2. Different letters indicate significant differences between treatments (gut sections and soil) for each group (GLM, Tukey's HSD test, $P < 0.05$). Mean ± S.E.M., $n = 3$.

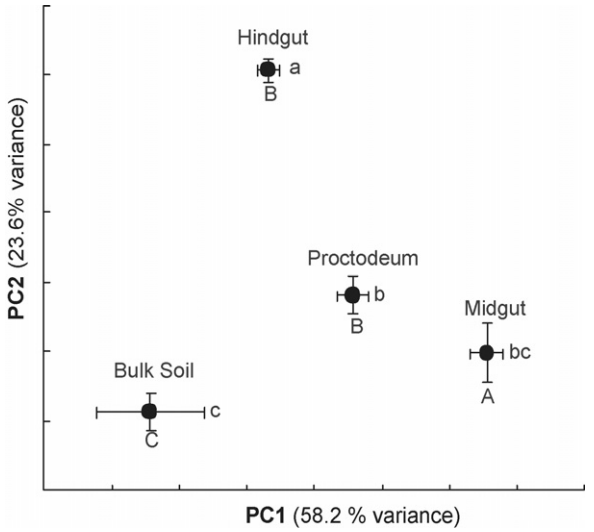

Fig. 2. Ordination plots of factor scores for the axis PC1 and PC2 extracted by principal component analysis conducted on the mole fraction of nine selected FAs extracted from the gut content of *L. terrestris* and bulk soil. PC1 was indicator of bacterial and fungal derived FAs relative enrichment. PC2 indicated a high mole fraction of short chain saturated FAs. Negative loadings in both axes indicate relative depletion in plant derived FAs already present in soil. Mean ± S.E.M., $n = 3$. Different letters (capital letters for the x-axis) indicate significant differences between treatments (gut sections and soil) for each axis (GLM, Tukey's HSD test, $P < 0.05$).

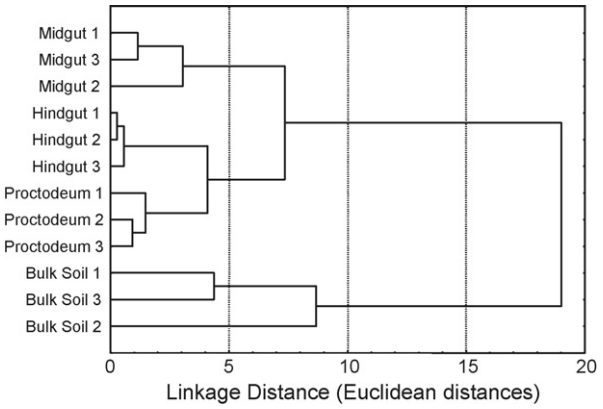

Fig. 3. Cluster analysis of the FAs of bacterial and fungal origin extracted from three sections of the gut content of the earthworm *L. terrestris* and from the bulk soil ($n = 3$). FAs ubiquitous and from uncertain origin (16:0, 18:0 and FAs ≥20C) were excluded from the analysis.

have originated from protozoan cells or from the earthworm, possibly from the gut wall cells. Finally, we observed a decrease ($F_{(3,8)} = 16.0$, $P < 0.001$) in the mole fraction of FAs derived from plants in the earthworm gut compared to bulk soil, suggesting that these FAs were digested during gut transit (Fig. 1).

The PCA conducted on the nine most informative known FAs biomarkers summarized the changes in the FAs profiles through the gut (Fig. 2). We excluded from the PCA typical animal PUFAs (such as 20:3ω6, 20:4ω6 and 22:5ω3) to avoid any possible interference in the analysis due to contamination from the earthworm gut wall. The main two axes extracted by the PCA explained 82% of the variance in the samples. Loadings along the PC1 (eigenvalue = 5.2) were highly positive for the bacterial (16:1ω7, 16:1ω5 and 18:1ω7) and the fungal biomarkers (18:1ω9, 18:2ω6 and 18:3ω6) included in the analysis; and moderately negative for the plant derived FAs (24:0). The PC1 axis was therefore an indicator of bacterial and fungal richness and low concentration of plant derived material. The highest values for this axis, in the midgut, were significantly different from those in the distal parts of the earthworm gut ($F_{(3,8)} = 22.5$, $P < 0.001$), and from the bulk soil (Fig. 2). The principal component PC2 (eigenvalue = 2.1) showed high positive loadings for 12:0 and 13:0, and also moderate negative loadings (factor loading = −0.67) for the plant derived FA. The value of PC2 in hindgut samples was significantly higher than in the other parts of the gut and bulk soil, indicating a relative enrichment in short chain saturated FAs, which was consistent with fermentative processes that likely occurs in this gut section.

To better identify changes in the FA profiles, the FAs ubiquitous in organisms and of uncertain origin were discarded and a cluster analysis was conducted only on bacterial and fungal derived FAs profiles (Fig. 3). Results from this cluster analysis summarized the changes in microbial derived FA through the earthworm gut. Profiles of the microbial community in bulk soil samples appeared more variable and clustered apart from the gut samples. In the group comprised of earthworm gut samples, the FA profiles in the hindgut were more closely related to those in the proctodeum than the midgut. Besides, the FA profiles in the hindgut showed the lower linkage distance, indicating a high similarity among replicates.

4. Discussion

4.1. FA concentrations in soil and the earthworm gut

The total FA concentration was greater in the earthworm gut, especially in the hindgut, than in bulk soil. There were also higher concentrations of Gram-positive and Gram-negative biomarker FAs in the

earthworm gut than in bulk soil (up to 500 times greater than some bacterial FAs present in bulk soil). Despite the high variability among replicates, differences in FA content between bulk soil and earthworm gut compartments were significant. These results are consistent with greater concentration of microorganisms in the earthworm gut than bulk soil, as has been reported for *L. terrestris* and other earthworm species (Parle, 1963; Márialigeti, 1979; Krištufek et al., 1992; Pedersen and Hendriksen, 1993; Horn et al., 2003). Anaerobic cultures from *L. rubellus* (Hoff.) contained 10–100 times more CFU in the earthworm gut than in bulk soil (Karsten and Drake, 1995, 1997; Ihssen et al., 2003), while epifluorescence microscopy demonstrated more bacterial cells in the gut of *L. terrestris* than bulk soil (Schonholzer et al., 1999), and an increase in bacterial numbers towards the distal part of the gut (Pedersen and Hendriksen, 1993; Fischer et al., 1995; Wolter and Scheu, 1999), although this latter observation could be a methodological artefact due to increased stainability of bacteria in the hindgut (Schonholzer et al., 1999).

We expect that the FA concentration in different sections of the earthworm gut is influenced by the digestive process, but this remains to be determined. In marine worms, the gut environment is affected by the partitioning of organic substrates along the gut. An extended retention time for the solubilized organic matter and a counter-current flow assisted digestion, with the dissolved component of the gut material flowing forward relative to the particulate component, have been proposed for marine deposit-feeding polychaetes (Jumars, 2000; Mayer et al., 2001). These mechanisms increase the dissolved organic contents into the gut lumen of marine worms, but it is not known whether similar processes occur in the gut of terrestrial worms. Although earthworms were maintained without added food and soil used for the culture bin was thoroughly homogenized, the selective ingestion of microbe-rich soil microsites could in part explain the observed difference in FA concentration between bulk and gut soil. We were unable to provide information on the chemical composition of gut soil, which could be compared to bulk soil, thus supporting or refuting the possibility of selective feeding. The small quantity of soil (about 200 mg) collected from each gut section of dissected earthworms within a reasonable time period (48 h) was sufficient only for the lipid analysis. Specific digestive mechanisms in earthworms and selective feeding are possibilities that warrant further investigation.

4.2. FA profiles and microbial community in soil and the earthworm gut

A substantial amount of FAs (about 15 mol% fraction) were found in the gut but were not detected in soil samples. The greatest concentrations of these FAs were found in the hindgut, including several bacterial and eukaryotic FAs. Clones matching acidobacteria, actinobacteria, firmicutes and β and γ-proteobacteria were found in the gut of *L. rubellus* (Singleton et al., 2003, 2004), and it was concluded that many of the intestine-associated phylotypes were not detected in either cast material or surrounding soil, suggesting that members of the gut-associated microbiota would be only rarely expelled. In addition, an increase in the numbers of both dormant soil bacteria and gut-specific taxa in the earthworm gut content was recently reported (Horn et al., 2003; Ihssen et al., 2003). The greater proportion of branched-chain FAs in the midgut than other gut compartments found in the present study may indicate an increase in the Gram-positive bacteria population after ingested soil passed through the gizzard. However, the prokaryote population in the gut seems to be mainly Gram-negative, which is consistent with earlier findings for other Lumbricidae species (Márialigeti, 1979).

Multivariate analysis indicated significant changes in the microbial-derived FA profiles of bulk soil and earthworm gut compartments, suggesting that changes in microbial community structure occurred during the transit through the gut of *L. terrestris* (Fig. 3). This is consistent with some rRNA studies that have shown greater numbers of Cytophaga-flavobacterium in the gut of *L. terrestris* in comparison to the surrounding soil (Schonholzer et al., 2002) and increasing numbers of proteobacteria (especially of β and γ-subgroups) in the distal part of the gut of this species (Fischer et al., 1995). The changes in FA profiles are as well in agreement to the small but significant differences in the Archaea and Bacteria communities of soil, midgut and casts recently suggested by means of molecular profiling of 16S rRNA genes (Egert et al., 2004).

Lower mole fractions of the plant derived FAs 22:0, 24:0, 20:2ω6 and 22:2ω6 were observed in the earthworm gut than bulk soil. This could indicate that these FAs were digested in the gut, but it seems unlikely that most of the plant derived FAs were digested by the time soil entered the midgut. On the other hand, it seems unlikely that *L. terrestris* selectively consumed soil and avoided plant debris, due to the known feeding habits of this earthworm. It will be necessary to investigate the origin and fate of these FAs further. We assumed that

these FAs were plant derived rather than from microeukaryotes. The two dienoic FAs (20:2ω6 and 22:2ω6) are direct products of the elongation of 18:2ω6 without any desaturation step; they are rarely found in animal tissues, but are abundant in some vegetal tissues of *Ranunculaceae* and other families (Aitzetmuller, 1996). The two saturated FAs (22:0 and 24:0) are frequently found in plant waxes, but they appear rarely in eukaryotic tissues and they are not a common constituent of other lipids. We also found that bulk soil contained large quantities of some LC-FAs, but there is relatively little information about these compounds in the literature, as they are not commonly analyzed (the GC run may terminate before LC-FAs elute when the extensively used MIDI System® procedure is applied).

The concentration and mole fraction of polyenoic FAs of eukaryotic origin in the earthworm gut were much greater than those in the bulk soil. This suggests an important input of eukaryotic-derived material into the gut. Prokaryote usually render less amount of EL-FAs than eukaryotic cells, because the eukaryotic cells contain both membrane lipids (polar El-FAs) and storage lipids (neutral EL-FAs), while prokaryote endogenous storage polymers (poly-β-hydroxyalkanoates) are not ester-linked lipids (White and Ringelberg, 1998; Boga and Brune, 2003). This observation could indicate contamination of our samples with FAs coming from the earthworm gut wall tissue, despite the careful sampling of the gut content. Absorptive cells in the gut wall of *L. terrestris* are known to possess reserves of neutral lipids (Stephenson, 1930). However, the characteristic protozoa biomarker 20:3ω6 (which appeared in high mol% in the earthworm gut samples) had not been previously observed as a FA constituent of earthworm tissues, although other FAs such as 30:5, 20:4ω15, 18:1ω7, 20:1ω9, 20:5ω3 and 20:4ω6 have been reported as major FAs of *L. terrestris* (Albro et al., 1992) and other earthworms species (Hansen and Czochanska, 1975; Petersen and Holmstrup, 2000; Enami et al., 2001). Additional studies are needed to verify the origin of these eukaryotic FAs. Protozoa has been found in the gut of earthworms (Miles, 1963; Cai et al., 2002), but their nature and function remains unclear. It has been suggested that they could provide essential nutritive factors to earthworms such as cholesterol (Wootton and Wright, 1962; Albro et al., 1993) and some amino acids (Pokarzhevskii et al., 1997). Whether the long chain PUFAs detected in the gut content of *L. terrestris* were a contamination from the tissue of the earthworm gut wall, or whether they belong to protozoa living in the earthworm gut is still unknown.

As far as we know, this is the first study of FA composition through the earthworm gut content. Here, we showed that significant changes in the FA concentration and composition occur at very small spatial scales inside the gut of the earthworm *L. terrestris*. These findings have implications for understanding earthworm digestion, and they suggest changes in the gut microbiome along the earthworm digestive tract. However, it is not known if differences in the FA profiles are a consequence of microbial community changes through the gut, or if they are caused by earthworm digestive processes, or both. Physiological conditions in the gut selectively favouring the growth (or the lysis) of some microorganisms likely contribute to the observed changes, but we cannot ignore other factors, such as selective feeding on biological micro hot spots or some phenomenon in earthworms' digestion that partitions organic substrates along the gut tube. Further studies on the structure and function of the microbial community at the earthworms' gut should be performed to improve our understanding of the interactions between earthworms and their gut microbiota, which ultimately influence ecosystem-level function such as organic matter decomposition, nutrient cycling and pedogenesis.

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