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ORIGINAL ARTICLE

Gut wall bacteria of earthworms: a natural selection process

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Earthworms and microorganisms are interdependent and their interactions regulate the biogeochemistry of terrestrial soils. Investigating earthworm–microorganism interactions, we tested the hypothesis that differences in burrowing and feeding habits of anecic and endogeic earthworms are reflected by the existence of ecological group-specific gut wall bacterial communities. Bacterial community was detected using automated ribosomal intergenic spacer analysis of 16S and 23S genes and ribotype data was used to assess diversity and community composition. Using soil and earthworm samples collected from adjacent wheat–barley and grass–clover fields, we found that the anecic *Lumbricus terrestris* and *L. friendi*, the endogeic *Aporrectodea caliginosa* and *A. longa* (classically defined as anecic, but now known to possess endogeic characteristics) contain ecological group-specific gut wall-associated bacterial communities. The abundance of specific gut wall-associated bacteria (identified by sequence analysis of ribotype bands), including Proteobacteria, Firmicutes and an actinobacterium, was ecological group dependent. A microcosm study, conducted using *A. caliginosa* and *L. terrestris* and five different feeding regimes, indicated that food resource can cause shifts in gut wall-associated bacterial community, but the magnitude of these shifts did not obscure the delineation between ecological group specificity. Using *A. caliginosa* and *A. longa* samples collected in six different arable fields, we deduced that, within an ecological group, habitat was a more important determinant of gut wall-associated bacterial community composition than was host species. Hence, we conclude that the selection of bacteria associated with the gut wall of earthworms is a natural selection process and the strongest determinant of this process is in the order ecological group > habitat > species.
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Introduction

Charles Darwin recognized and described the importance of earthworm activity in soils (Darwin, 1881). Earthworms (class Oligochaeta) comprise approximately 800 genera and 8000 species that account for up to 90% of invertebrate biomass present in soil (Edwards, 2004). They are ubiquitous, abundant and highly productive organisms; they are 'keystone species' in soil food webs and 'ecosystem engineers' in soils (Jones *et al.*, 1994; Brown *et al.*, 2000). Earthworms influence primary soil functions and processes, such as soil structure formation, soil carbon dynamics and biogeochemical cycles (Brown and Doube, 2004; Lavelle *et al.*, 2004). The successful management and exploitation of earthworm bioresources has the potential to deliver significant economic and environmental benefits, especially in light of global concerns regarding sustainable land use, food security and climate change.

Earthworms affect ecosystem structure and function directly by ingesting, altering and mixing organic residues and mineral soil. Through these actions, they change the structure, chemistry and biology of soil (Lavelle *et al.*, 2004). European earthworms are classified into three ecological groups based on their distinct feeding and burrowing habits (Bouché, 1977). Stable isotope analysis has confirmed and refined conventional ecological classification systems (Briones and Schmidt, 2004). Epigeic earthworms live above mineral soil, rarely form burrows and feed preferentially on plant litter. Endogeic earthworms forage below the surface soil, ingest large quantities of mineral soils and humified material, and they build ramified, predominantly horizontal, burrows. Anecic earthworms build permanent, vertical burrows deep into the mineral soil layer, and they come to the surface to feed on partially decomposed plant litter, manure and other organic residues. The ecological groups of some

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common, but not all earthworm species, are clearly established. For example, *Aporrectodea caliginosa* is an endogeic and both *Lumbricus terrestris* and *L. friendi* are anecic species (Bouché, 1977; Lee, 1985; Schmidt *et al*., 1997; Schmidt *et al*., 2004).

Differences in earthworm digestion and assimilation processes suggest the possible existence of ecological group-specific gut microbiota (Lavelle and Spain, 2001). Although the microbial profile of the gut content is akin to that of soil and feed resources (Egert *et al*., 2004; Drake and Horn, 2007; Knapp *et al*., 2008, 2009), it is not a coincidental combination of the microorganisms present in soil (Sampedro and Whalen, 2007). The evolutionary relationship between earthworm burrowing and feeding habits and the gut microbial community has not been defined. However, based on studies conducted on insects and faunal gut-associated microbial communities (Zientz *et al*., 2004; Dale and Moran, 2006; Ladygina *et al*., 2009), we can expect the microbial profile of the gut to be an important determinant of earthworm metabolism. Diet, host anatomy and phylogeny have been shown to influence the composition of microbiota within the gut of carnivores, herbivores and omnivores, including humans and primates (Ley *et al*., 2008). However, there is no information available regarding the comparative microbial community composition in different earthworm ecological groups or the association between gut microbiota biodiversity and ecological groups.

This study analysed the relationship between bacterial community tightly associated with the gut wall and earthworm ecological groups and environment. Bacteria were discriminated using automated ribosomal intergenic spacer analysis (ARISA) of the intergenic spacer (IGS) region between bacterial 16S-23S rRNA genes. Earthworms and soil collected from the field (from pairs of adjacent arable and pasture fields) and a microcosm study (where earthworms were subjected to different food resources) were analysed to determine the relationship between gut wall bacterial community and both earthworm ecological groups and species. Earthworm and soil samples from three geographical locations, incorporating field sites under different management practices and agricultural regimes, were analysed to determine the relative impact of habitat and species on gut wall-associated bacterial diversity.

Methods

Earthworm ecological groups and field sampling

The earthworm (Lumbricidae, Annelida) species sampled from field sites represented two ecological groups, namely the anecics *L. terrestris* and *L. friendi*, and the endogeics *A. caliginosa* and *A. longa*. *A. longa* was traditionally considered an anecic earthworm, but recent isotopic and feeding and burrowing behaviour experiments suggest that it can exhibit endogeic features (Schmidt *et al*., 2004; Briones *et al*., 2005; Eisenhauer *et al*., 2008). These four earthworm species are common inhabitants of temperate agro-ecosystems (Edwards, 2004). Earthworms and soils were collected in April 2006 from six agricultural fields from three locations in Ireland, from pairs of two adjacent fields (arable and pasture) per location. The agricultural management practices and the physico-chemical properties of the soils from these fields are presented in the Supplementary Table S1. Three composite soil samples (each composite sample composed of five samples at 0–20 cm depth from five random earthworm sampling spots) from each field were used for physico-chemical analysis. All physico-chemical tests are described in the Supporting Information (SI) Text. Earthworms (4–10 individuals per species) were immediately hand-sorted and preserved in 100% ethanol (in the field) and soil samples (0–20 cm soil depth) were collected from the same spots and immediately placed in an icebox (Thakuria *et al*., 2009). Earthworms were identified to species level (Sims and Gerard, 1999). Earthworm gut walls were isolated as previously described (Thakuria *et al*., 2009).

Microcosm study

The microcosm units (35 cm high, 11 cm diameter), the soil used therein and its properties are described in the SI Text. Microcosm units were incubated in a controlled environment chamber (constant darkness, $17.4 \pm 0.5\ ^{\circ}C$). Microcosm units were watered (to 95% water-holding capacity) and left undisturbed for 48 h before the addition of adult earthworms. Endogeic (*A. caliginosa*) and anecic (*L. terrestris*) earthworms were collected from the grass–clover field from where microcosm soil was collected (see SI Text). Earthworms were collected using a mustard oil extraction method (allyl isothiocyanate 0.05% $v\,v^{-1}$; Sigma-Aldrich, Steinheim, Germany) and identified to species level as previously described (Sims and Gerard, 1999). Earthworms were stored in soil of origin at 17 °C for 24 h before their addition to microcosm units. One individual per species was added to each microcosm unit, and units were incubated as above for a further 48 h before the addition of feeds. Feeding treatments were control (no litter material and no mineral N), maize litter, maize litter plus mineral N, maize litter plus clover litter, maize litter plus clover litter plus mineral N (see SI text). Microcosms were incubated as above, with weekly watering and randomization of units. After 45 days, earthworms were hand-sorted and immediately preserved in 100% ethanol. All introduced individuals of the species *L. terrestris* and *A. caliginosa* were alive on recovery. Soil samples (0–20 cm depth) were also collected from each microcosm. This experiment included six replicate microcosm units per feeding

treatment and earthworms and soil of each microcosm were subsequently analysed.

Earthworm dissection and gut nucleic acid extraction
The procedures for earthworm dissection and extraction of gut wall DNA and soil DNA have been previously described (Thakuria *et al.*, 2008, 2009). The absorption spectrum of gut nucleic acid extracts (230–280 nm) was determined using a Nanodrop ND-1000 spectrophotometer (Labtech International, Eastbourne, UK) according to manufacturer's instruction. The quality of gut and soil DNA extracts was comparable; the 260/230 nm values ranged from 1.74 to 1.85 and the 260/280 nm values ranged from 1.80 to 1.95. Gut wall nucleic acid was visualized by electrophoresis and was quantified, as previously described (Thakuria *et al.*, 2008). DNA yields ranged from 6 to 15 μg 0.1 g^{-1} gut wall tissue and from 30 to 40 μg g^{-1} dry soil.

Automated ribosomal intergenic spacer analysis
ARISA fingerprinting was used to determine bacterial communities associated with gut wall and soil DNA samples. The partial 16S and 23S rRNA gene and the IGS region was amplified using primer pair ITSF/ITSReub (Cardinale *et al.*, 2004). The 5′ end of the forward primer ITSF was labelled with 6-carboxyfluorescein dye (Applied Biosystems, Warrington, UK). All ARISA amplification reactions were performed using Platinum high-fidelity *Taq* DNA polymerase (Invitrogen, Glasgow, UK). Bacterial DNA amplification reaction (25 μl) contained 10 ng DNA of gut wall extracts as template DNA and other PCR components and reaction conditions were as previously described (Thakuria *et al.*, 2008). PCR products were diluted by mixing with PCR grade water (Gibco, Glasgow, UK) to obtain 500 ng amplified DNA per microlitre of diluted PCR product. One microlitre (500 ng) of amplified DNA was mixed with 0.3 μl of internal size standard (LIZ-1200; Applied Biosystems) and 8.7 μl HiDi formamide (Applied Biosystems). This mix was denatured at 95 °C for 3 min and immediately placed on ice for at least 5 min before loading on an ABI 3130 × l Genetic Analyzer (Applied Biosystems, CA, USA) equipped with a 16 channel capillary array (50-cm long) filled with POP7 polymer (Applied Biosystems). The run duration was 8000 s and running conditions were as follows: 60 °C oven temperature, 15 s injection time, 1.6 kV injection voltage and 8.0 kV run voltage.

Duplicate ARISA fingerprints were generated for each DNA extract. Ribotype size and abundance of ribotypes were determined using the GeneMapper software 4.0 (Applied Biosystems), as previously described (Thakuria *et al.*, 2009). Output ribotype area abundance values were exported to the PRIMER (Plymouth Routines in Multivariate Ecological Research) v.6.1.9 software (PRIMER-E Ltd., Plymouth Marine Laboratory, UK) where they were transformed into relative abundances and binary formats. Binary data were used to determine ribotype reproducibility; a reproducible ribotype was defined as one that was present in the majority ($\geqslant$66%) of samples for a given earthworm species or soil. Ribotype relative abundance data were used to calculate diversity indices (Margalef's richness, Pielou's evenness and Shannon's diversity using $\mathrm{Log_e}$ (H') indices) and diversity indices were compared by univariate analysis (Thakuria *et al.*, 2009). Bray–Curtis resemblance matrices were calculated using ribotype relative abundance data. One-way analysis of similarity (ANOSIM) was performed on each Bray–Curtis resemblance matrix (incorporating 999 permutations for R statistics) to determine the significance of differences between earthworm species from different habitats or under different feeding conditions in the microcosm experiment in terms of the derived ARISA profiles (pair-wise comparisons). Each Bray–Curtis resemblance matrix was plotted in two dimensions by non-metric multidimensional scaling (NMDS) ordination and hierarchical cluster generated using each Bray–Curtis resemblance matrix was superimposed on the NMDS plot to form ellipses at arbitrary resemblance levels of slices drawn through the dendrograms (Thakuria *et al.*, 2009). Stress (goodness of fit of the NMDS plot) was calculated as described by Kruskal (1964); a stress level of $\leqslant$0.1 corresponds to an ideal ordination (Clarke, 1993).

Isolation, sequencing and analysis of relatively abundant RISA products
The partial 16S and 23S rRNA genes and the IGS region were amplified as described above for ARISA analysis (except unlabelled primers were used). Products were analysed by non-denaturing polyacrylamide gel electrophoresis (PAGE) and bands of interest were excised from the gels, cloned and sequenced (bidirectional) as described in the SI Text. The consensus 16S-ITS-23S (excluding primer binding sites) sequence obtained for each band was subjected to BLASTn analysis (Altschul *et al.*, 1990) using the nucleotide database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). The closest relatives were determined based on the identity of the organisms with the most homologous 16S-ITS-23S sequences. The 16S-IGS-23S rRNA sequences have been deposited in GenBank under accession numbers FJ712630 through FJ712656.

Results

Relationship between bacterial community tightly associated with the gut wall and earthworm ecological group
The bacterial community tightly associated with the gut wall of earthworms and within soil samples taken from both an arable field and an adjacent

pasture field (that is a wheat–barley and a grass–clover field located at Johnstown Castle Estate, Wexford, Co. Wexford; Supplementary Table S1) was determined. ARISA analysis was conducted for two earthworm species commonly found in both fields (*A. caliginosa* and *A. longa*), for *L. terrestris* found in the arable and for *L. friendi* found in the pasture field ($n=4$ per species per field site) (Supplementary Table S1). Earthworm gut walls harboured less bacterial diversity as the soils from which they originated, and all bacterial ribotypes detected in gut wall samples were present in the associated soil sample (Table 1; Supplementary Figures S1 and S2). The ranking of earthworm species with respect to the gut wall-associated bacterial diversity varied depending on which of the two adjacent fields they originated from (see diversity indices in Table 1). Gut wall-associated bacterial ribotypes from the endogeic species *A. caliginosa* and the species *A. longa* were more unevenly distributed than those from the anecic species *L. terrestris* and *L. friendi* or those from soils (Pielou's evenness indices; Table 1).

Each earthworm species harboured distinct gut wall-associated bacterial community compositions, except those of *A. caliginosa* and *A. longa* (pair-wise ANOSIM comparisons: $R=0.68$ in wheat–barley field and $R=0.45$ in grass–clover field; Global $R=0.84$; $P<0.001$; Supplementary Table S2). NMDS and hierarchical cluster analysis (based on bacterial ribotype abundance data) showed that the bacterial communities from well-defined endogeic (*A. caliginosa*) and anecic (*L. terrestris* or *L. friendi*) species formed two distinct groups within each field, and these groups were distinct from the group formed by bacteria from associated soil; these three groupings shared $\leqslant 25\%$ similarity (Figure 1). Remarkably, bacteria associated with the gut walls of the species *A. longa* grouped with those from the endogeic species *A. caliginosa* ($\geqslant 75\%$ similarity; Figure 1). Within the NMDS plot, the distance between ecological groupings was greater than the distance between an earthworm species in terms of its two fields of origin.

The relative abundance of 27 bacterial ribotypes was high in the gut wall of one or more of the earthworm species analysed (representing $\geqslant 1.0\%$ of the total fluorescence units). Relative to other bacteria, all were significantly less abundant in soil than in the gut wall of at least one earthworm species isolated from that soil ($P<0.01$; results not shown). These ribotypes were retrieved from the non-denaturing PAGE gel (see Supplementary Figure S3) and re-amplified by ARISA-PCR. Inserts (ARISA-PCR products obtained from PAGE gel) were cloned and both ARISA-PCR of transformed colonies and restriction digestion of the transformed plasmid using *Not* 1 confirmed that inserts of expected size had been cloned (results not shown). These ribotypes were sequenced and, based on the origin of their homologues (BLASTn analysis; Supplementary Table S3), we tentatively identified the 27 bacteria, including 9 whose relative abundance on the gut walls was ecological group dependent ($P<0.01$; Figure 2). A δ-proteobacterium (strain DTE 13), two β-proteobacterium (strains DTE19 and DTE23), an unidentified bacterium (strain DTE15) and an actinobacterium (*Rhodococcus* sp.; strain DTE17) were of relatively high abundance in *A. caliginosa* and *A. longa*, compared with other earthworm species or soil, irrespective of field of origin (that is arable or pasture). Three Firmicutes (strains DTE3, DTE5 and DTE8) and an unidentified bacterium (strain DTE7) were more of relatively high abundance in *L. terrestris* and *L. friendi,* as compared with either *A. caliginosa*, *A. longa* or soil, irrespective of field of origin (Figure 2).

Ecological group is more important than food resource availability in determining gut wall bacterial diversity

A microcosm study was conducted to determine the influence of the availability of different food

Table 1 The reproducible ribotype number and diversity indices of bacteria associated with the gut walls of earthworms and with soils from a wheat–barley and a grass–clover field (Johnstown Castle), as determined based on data generated by ARISA analysis

Earthworm species and soils	*Wheat–barley field*[a,b]				*Grass–clover field*[a,b]			
	Reproducible ribotype numbers	*Margalef's richness*	*Pielou's evenness*	*Shannon's diversity*	*Reproducible ribotype numbers*	*Margalef's richness*	*Pielou's evenness*	*Shannon's diversity*
Aporrectodea longa	144	11.10b	0.70a	3.66b	70	6.06a	0.49a	2.12a
A. caliginosa	133	10.20b	0.67a	3.28b	67	5.05a	0.49a	2.05a
Lumbricus terrestris	92	7.06a	0.83b	2.74a	NF	NF	NF	NF
L. friendi	NF	NF	NF	NF	92	7.01a	0.75b	3.38b
Soil (0–20 cm depth)	201	15.40c	0.80b	4.22c	197	15.00b	0.78b	4.12c

Abbreviation: ARISA, automated ribosomal intergenic spacer analysis.
Reproducible ribotypes were defined as those that were common to at least three of the four ARISA profiles (each ARISA profile generated from individual earthworm). Diversity indices were calculated based on the relative abundances of ribotypes.
[a]NF denotes the fact that this species was not found in this field.
[b]Values within a column that differ significantly ($P<0.01$, based on Tukey's HSD test within one-way analysis of variances) are followed by different letters.

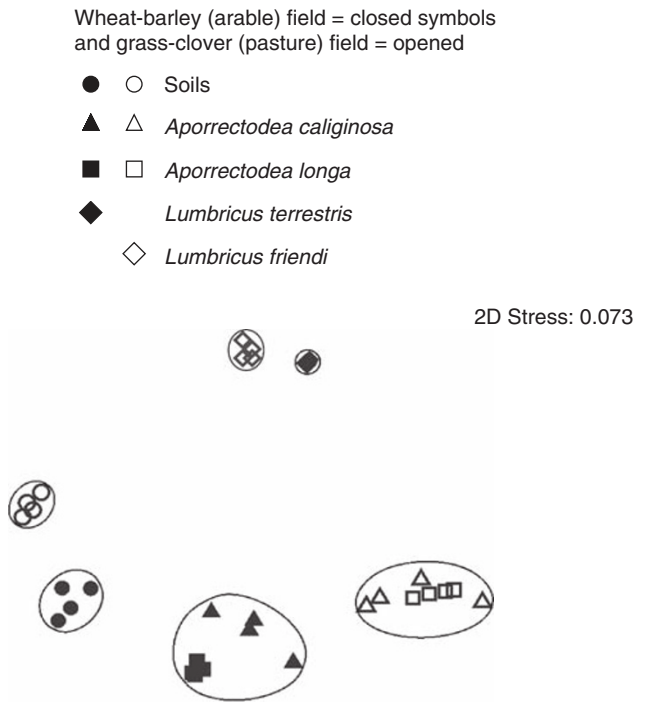

Figure 1 Variability of the bacterial community composition tightly associated with the gut walls of common endogeic and anecic earthworms and in their soil of origin. Ribotype abundance data was derived by automated ribosomal intergenic spacer analysis (ARISA) of DNA extracted from the gut walls of four earthworm species and soils (0–20 cm depth) from the earthworm sampling spots. Non-metric multidimensional scaling ordination plot was derived using a Bray–Curtis resemblance matrix generated using bacterial ribotype abundance data. Closed symbols represent earthworms and soils from a wheat–barley field, and opened symbols represent earthworms and soils from an adjacent grass–clover field (Johnstown Castle). Results are based on ribotypes detected in at least ⩾75% of samples analysed per earthworm species/soil samples per site. Ellipses represent superimposed hierarchical clusters (similarity level 75%), deduced using group-average linking based on the Bray–Curtis resemblance matrix.

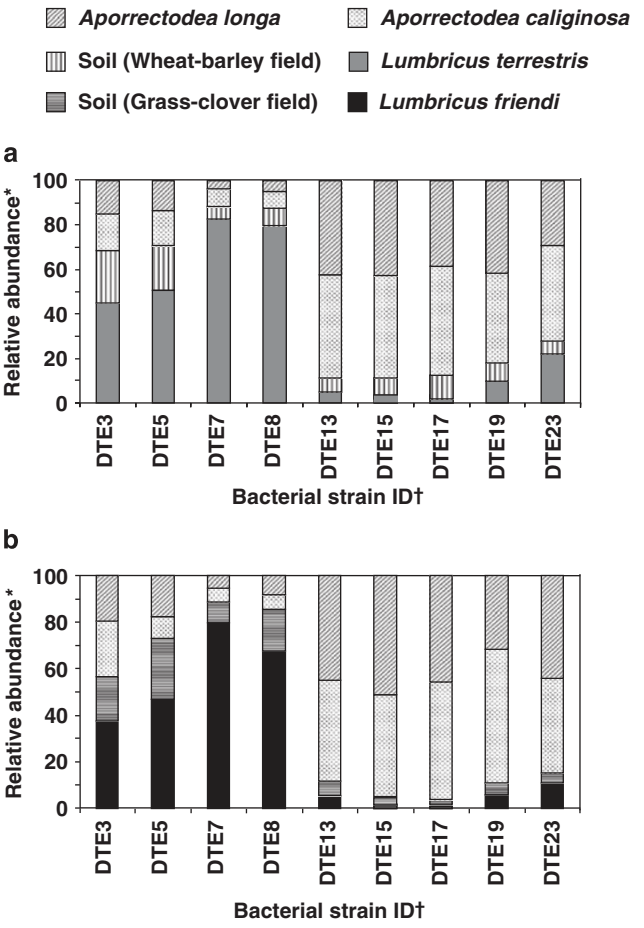

Figure 2 The abundance of nine bacterial ribotypes tightly associated with the gut walls of common endogeic and anecic earthworms and in the soils collected from (**a**) wheat–barley field and (**b**) grass–clover field (Johnstown Castle). Ribotypes were detected by automated ribosomal intergenic spacer analysis (ARISA) of the DNA extracts obtained for the gut walls of five earthworm species and soils (0–20 cm depth). *Relative abundance was estimated as the fluorescence units of a given ribotype, expressed as a percentage of the total fluorescent units obtained for the all ribotypes within an ARISA profile. Results are based on ribotypes detected in at least ⩾75% samples analysed per earthworm species/soil sample per site. †The closest relatives attributed to the bacteria, based on 16S-ITS-23S sequence homology (Supplementary Table S3): DTE3, Firmicute/*Lysinibacillus*; DTE5 and DTE8, Firmicutes/*Bacillus*; DTE7 and DTE15, phylum/genus unknown; DTE13, δ-proteobacterium/genus unknown; DTE17, actinobacterium/*Rhodococcus*; DTE19, uncultured β-proteobacterium/genus unknown; DTE23, β-proteobacterium/*Acidovorax*. Differences in ribotype abundance between earthworm ecological groups (*Aporrectodea longa* and *A. caliginosa* versus *Lumbricus terrestris* or *L. friendi*), and between earthworm species and soil samples were significant ($P<0.01$; Kruskal–Wallis *H*-test).

resource types (plant litter types, maize versus clover, with or without mineral N supplements) on the gut wall-associated bacterial community of the endogeic earthworm *A. caliginosa* and the anecic earthworm *L. terrestris.* NMDS and hierarchical cluster analysis (based on ARISA bacterial ribotype abundance data) showed that variation in food resources resulted in a shift in the gut wall bacterial community composition within both earthworm ecological groups after 45 days (Figure 3). These results disclosed that species was a stronger determinant of variation in bacterial community than was the supplied food resource type. The bacterial community spacing between *A. caliginosa* and *L. terrestris* was always greater than any shift within an ecological group due to change of supplied food resource type (clustering between ecological groups at ⩽50%). For each ecological group, the bacterial communities formed three sub-clusters based on food resource types (⩾75% similarity within each cluster). These three sub-clusters were distinguished by the presence of either maize litter or maize plus clover litter in the microcosm (Figure 3). Therefore, we concluded that these two earthworm ecological groups formed relationships with distinct gut wall-associated bacterial communities, and that relationship can be altered to some degree by the supplied food resources.

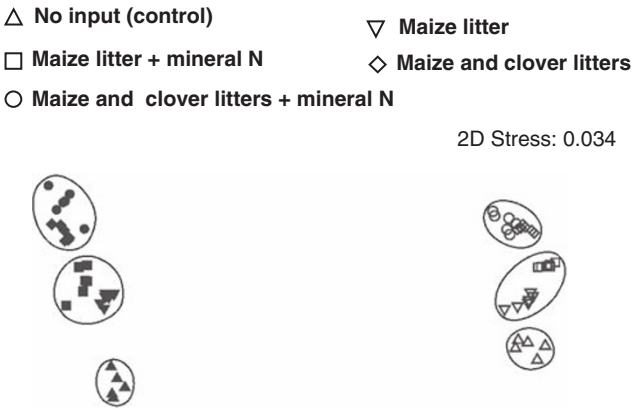

Figure 3 Microcosm study: influence of food resource type and availability on the bacterial community composition tightly associated with the gut wall of common earthworms *Aporrectodea caliginosa* and *Lumbricus terrestris*. Ribotype abundance data were derived by automated ribosomal intergenic spacer analysis (ARISA) of the DNA extracted from the gut contents of *A. caliginosa* (open symbols) and *L. terrestris* (closed symbols) reared under different feeding conditions. A non-metric multidimensional scaling (NMDS) ordination plot was derived using the Bray–Curtis resemblance matrix generated using bacterial ribotype abundance data. Results are based on ribotypes detected in at least four of the six earthworm samples analysed per species (one per microcosm unit). Ellipses represent superimposed hierarchical clusters (similarity levels $\geqslant$75%), deduced using group-average linking based on the same Bray–Curtis resemblance matrix.

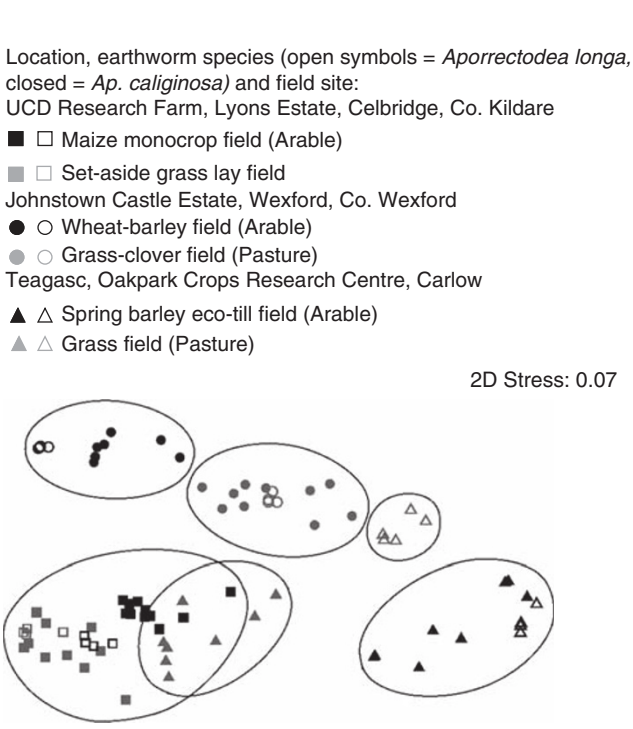

Figure 4 Influence of habitat on the bacterial community composition tightly associated with the gut walls of the common earthworms *Aporrectodea caliginosa* and *A. longa*. Earthworms were collected from six fields and ribotype abundance data were derived by automated ribosomal intergenic spacer analysis (ARISA) of DNA extracted from the gut walls. The non-metric multidimensional scaling ordination plot was derived using the Bray–Curtis resemblance matrix generated using bacterial ribotype abundance data. Results are based on ribotypes detected in at least 75% of 4–10 samples analysed per earthworm species/soil sample per site. Ellipses represent superimposed hierarchical clusters (similarity levels $\geqslant$50%), deduced using group-average linking based on the Bray–Curtis resemblance matrix.

Species level is the phylogenetic branch point at which habitat significantly impacts upon the gut wall bacterial community within an ecological group

Results from the field study suggested that, within an ecological group, field site was a more important determinant of gut wall bacterial community structure than was host species (Figure 1). Using *A. caliginosa* and *A. longa* earthworms collected from all three locations and the six field sites listed in Supplementary Table S1 ($n = 4$–10 per species per field site), we tested the hypothesis that environment (habitat and food type) significantly impacted upon ecological group-dependent gut wall-associated bacterial community at host species level. NMDS and hierarchical cluster analysis (based on ARISA bacterial ribotype abundance data) revealed that habitat was a more important determinant of bacterial diversity than host species (Figure 4). The gut wall-associated bacterial communities of *A. caliginosa* and *A. longa* generally formed an ensemble ($\geqslant$50% similarity) within each field; the exception was that this did not occur for earthworms sampled from the permanent pasture field at Oak Park (Figure 4). Within each field ensemble, the difference between the gut wall bacterial communities of these two earthworm species was non-significant, as determined by ANOSIM (R values <0.93 ($=$Global R), $P<0.01$; Supplementary Table S4). The only exception was the permanent pasture field at Oak Park. Habitat did not impact upon the incidence or abundance of the most prevalent bacterial ribotypes detected in both host species (results not shown).

Discussion

This study showed that common species of earthworm ecological groups foster the development of distinct gut wall-associated bacterial communities and that the relative abundance of specific bacteria within the gut wall, including Proteobacteria, Firmicutes and an actinobacterium, is ecological group specific. Our results show that food resource type and habitat can cause bacterial community shifts at the gut wall, but the magnitude of these shifts does not obscure the delineation between ecological group-specific gut wall bacterial communities. Analysis of more genera of earthworms will determine whether genus mirrors ecological groups with respect to differences in gut wall-associated microbiota. However, it is clear from this study that ecological group outweighed habitat and that habitat outweighed species with respect to its influence on bacterial communities tightly associated with the

gut wall of earthworms. In a recent study, Ladygina *et al*. (2009) showed that grassland soil nematodes harbour feeding group-specific gut bacterial diversity.

The tenacity of earthworms for specific food types reflects their metabolic capacity (Brown and Doube, 2004). Physical, physiological and biochemical properties dictate the metabolic capacity of the earthworm gut (Drake and Horn, 2007). In mammals, gut morphology significantly influences bacterial community compositions (Ley *et al.*, 2008). Although the complexity of the earthworm gut is relatively low, ecological groups do differ in their gut morphology and gut transit time for passage of ingested material. For example, anecic earthworms have a longer gut, a simpler typhlosole with less folding, a longer gut transit time and sharper gut contractions, as compared with endogeics (Wu, 1939; Perel, 1977; Breidenbach, 2002). Differences in gut morphology, folding and contractions most likely contribute to the establishment of distinct bacterial communities across the earthworm ecological groups. Bacteria make a significant contribution to the biochemical activity in the gut of organisms (Lattaud *et al.*, 1998) and it is likely that differences in diet among earthworm ecological groups lead to the establishment of different bacterial communities.

All bacteria found within earthworms were also detected within the associated soil samples. This fact and the nature of this study means that it is not possible to determine whether bacteria tightly associated with the gut wall share a symbiotic or a mutualistic metabolic relationship with their host. Bacteria may be selected from the ingested material because they confer the host with a metabolic advantage (for example vitamins, minerals, digestive enzymes) and they could form an opportunistic association with the gut wall. Alternatively, some gut wall bacteria may represent true symbionts that form stable populations and have a critical function in host nutrition by enhancing metabolite acquisition, synthesis or catabolism (Moran, 2006). For instance, bacterial symbionts enable marine Oligochaetes exploit unusual energy sources present in their habitat (Dubilier *et al.*, 2008). However, identified bacterial symbionts of invertebrates usually reside within more specialized compartments or organs. Earthworms of the family Lumbricidae harbour specific and stable populations of *Acidovorax*-like bacteria within their excretory organs, the nephridia and these symbionts are selectively recruited through embryonic duct during embryogenesis (Davidson and Stahl, 2008). In addition to *Acidovorax*-like bacteria, these authors also observed a mixed population of bacteria composed primarily of γ- and β-Proteobacteria cell types that interact with the embryos externally and internally during the full course of development, and ultimately fill the gut lumen near the end of development before hatching. So, we hypothesize that the gut lumen bacterial community may continue to reside as tightly associated gut wall bacteria in the later juvenile stages. This might be the reason why the members of the Proteobacteria detected in the gut walls of both endogeics and anecics earthworm species were relatively abundant. As is the case for other annelids–symbionts relationships (Bright and Giere, 2005), there is possibility that horizontal transmission might provide evidence of strong fidelity between tightly associated gut wall bacteria and Lumbricidae.

The importance of habitat in the formation of gut wall-associated bacterial communities within and across species supports the hypothesis that the acquisition of a new diet is a fundamental driver for the evolution of new species (Moran, 2006). The fact that diet quality influences the microbial community composition of earthworm gut contents in the short term was previously verified (Egert *et al.*, 2004; Knapp *et al.*, 2008, 2009). The multidimensional soil habitat is composed of an immensely heterogeneous distribution of habitat types and food resources (Ritz *et al.*, 2004). Species are known to adapt themselves according to habitat type to reduce the limiting effects of biotic and abiotic factors of existence (Odum, 1971). In doing so, earthworms adjust themselves in terms of their diets, and diet quality and availability are habitat specific. This may be an explanation why the impact of habitat is reflected at host (earthworm) species level in terms of their gut wall-associated bacterial community.

The occurrence of gut wall bacteria of earthworms reported in this study was also observed by other workers in various earthworm species on different occasions. Members of the Firmicutes were found in the intestinal tissues of earthworm species *L. terrestris*, *Octolasion cyaneum*, *Lumbricus rubellus* and *Onychochaeta borincana* (Jolly *et al.*, 1993; Singleton *et al.*, 2003; Valle-Molinares *et al.*, 2007). *Bacillus* species and strains might aid earthworms by mineralizing phosphate and reducing nitrogenous compounds (Ihssen *et al.*, 2003; Wan and Wong, 2004). A few of the gut wall-associated bacteria that were relatively abundant in both the endogeic and anecic earthworm species were closely related to *Bradyrhizobium*, *Mycobacterium*, *Acidovorax* and *Streptomyces* strains. The presence of these species may be functionally significant in terms of both C and N metabolism. *Bradyrhizobium* are known to colonize guts of many soil dwelling animals, including earthworms (Citernesi *et al.*, 1977). *Mycobacteria* are known to use humic and fulvic acids in soils (Kirschner *et al.*, 1999), and *Mycobacterium avium* and *M. gastri* strain were previously isolated from *L. rubellus* guts (Fisher *et al.*, 2003). *Acidovorax* bacteria are well-known nephridial symbionts of many earthworm species, and it was postulated that they could be important in protein degradation during nitrogenous excretion by earthworms (Schramm *et al.*, 2003; Davidson and Stahl, 2006). *Streptomyces* are believed to be involved in

the assimilation of hemicellulose, xylans and xylose present in ingested crop residues, because the majority of *Streptomyces* found in soil possess glucose isomerase activities (Killham and Prosser, 2007). Bacteria with homology to *Geobacter sulfurreducens* and a *Rhodococcus* sp. were more abundant (relative to other bacteria) in the gut walls of endogeic as compared with anecic species. This might reflect the ability of endogeics to use more complex stabilized soil humic substances than do anecic species (Briones *et al.*, 2005). *Geobacter* species can completely oxidize organic compounds to carbon dioxide using Fe(III) as the electron acceptor (Lovley *et al.*, 1993). (Verma *et al.*, 2006) isolated a chlorinated hydrocarbon-degrading *Rhodococcus* sp. from the gut of an Indian earthworm, *Metaphire posthuma*.

The burrowing and feeding behaviour of *A. longa* was more typical of an endogeic rather than an anecic earthworm species (Eisenhauer *et al.*, 2008), although this species was traditionally considered an anecic species (Bouché, 1977). Its classification as an endogeic species was also supported by recent isotopic studies (Schmidt *et al.*, 2004; Briones *et al.*, 2005) and by our findings. Endogeic species are important ecosystem engineers, because they facilitate the mixing of surface and subsurface soil layers through their continuous horizontal burrowing activities. Therefore, the reclassification of *A. longa* as an endogeic species would have significant implications regarding our perception of its influence on soil structure formation and C sequestration, particularly given its large body size and hence its ingestion capacity. Changes in soil structure and C sequestration can significantly alter the soil biological functions and hence affect organic matter decomposition (Byers *et al.*, 2006).

In conclusion, this study showed that the development of the gut wall-associated bacterial community in some earthworm species is a process of natural selection. The strongest determinant for selection of the gut wall-associated bacterial community is in the order of ecological group > habitat > species. All members of the gut wall-associated bacteria were detected in soil and their relative abundances on gut walls were influenced by habitat (quality and availability of food resources); this has significant implications, in that it suggests that perturbation of the soil ecosystem could impact on earthworm gut wall-associated bacterial community composition and hence on earthworm ecology and functioning. Having determined that commonly found members of earthworm ecological groups house distinct gut wall-associated bacterial communities, the challenge is to determine the functional significance of the bacteria, particularly those whose relative abundance is ecological group dependent. Understanding the composition and function of the earthworm gut wall-associated bacterial community will help designing apt management practices for sustainable agriculture and other land uses. By facilitating the formation of an appropriate gut wall-associated bacterial community, we will maximize our ability to exploit benefits of earthworms for sustainability of soil ecosystem at local, regional and global scales.

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References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. (1990). Basic local alignment search tool. *J Mol Biol* **215**: 403–410.

Bouché MB. (1977) In: Lohm U and Persson T (eds). Soil Organisms as Components of Ecosystems: Strategies lombriciennes. *Ecol Bull* **25**: 122–132.

Breidenbach J. (2002). Normalanatomie und histologie des Lumbriciden *Lumbricus terrestris* L. (Annelida, Oligochaeta). PhD Thesis, University of Muenster: Germany, pp 253.

Bright M, Giere O. (2005). Microbial symbiosis in Annelida. *Symbiosis* **38**: 1–45.

Briones MJI, Garnett MH, Piearce TG. (2005). Earthworm ecological groupings based on ^{14}C analysis. *Soil Biol Biochem* **37**: 2145–2149.

Briones MJI, Schmidt O. (2004). Stable isotope techniques in studies of the ecological diversity and functions of earthworm communities in agricultural soils. *Recent Res Dev Crop Sci* **1**: 11–26.

Brown GG, Barois I, Lavelle P. (2000). Regulation of soil organic matter dynamics and microbial activity in the drilosphere and the role of interactions with other edaphic functional domains. *Eur J Soil Biol* **36**: 177–198.

Brown GG, Doube BM. (2004). Functional interactions between earthworms, microorganisms, organic matter, and plants. In: Edwards CA (ed). *Earthworm Ecology*. CRC Press LLC: Boca Raton, FL, pp 213–239.

Byers JE, Cuddington K, Jones CG, Talley TS, Hastings A, Lambrinos JG *et al.* (2006). Using ecosystem engineers to restore ecological systems. *Trends Ecol Evol* **21**: 493–500.

Cardinale M, Brusetti L, Quatrini P, Borin S, Puglia AM, Rizzi A *et al.* (2004). Comparison of different primer sets for use in automated ribosomal intergenic spacer analysis of complex bacterial communities. *Appl Environ Microbiol* **70**: 6147–6156.

Citernesi U, Neglia R, Serriti A, Lepidi AA, Filippi C, Bagnoli G *et al.* (1977). Nitrogen fixation in the gasteroenteric cavity of soil animals. *Soil Biol Biochem* **9**: 71–72.

Clarke KR. (1993). Non-parametric multivariate analyses of changes in community structure. *Aust J Ecol* **18**: 117–143.

Dale C, Moran NA. (2006). Molecular interactions between bacterial symbionts and their hosts. *Cell* **126**: 453–465.

Darwin C. (1881). *The Formation of Vegetable Mould Through the Action of Worms, With Observations of Their Habits*. Murray: London, pp 328.

Davidson SK, Stahl DA. (2006). Transmission of nephridial bacteria of the earthworm *Eisenia fetida*. *Appl Environ Microbiol* **72**: 769–775.

Davidson SK, Stahl DA. (2008). Selective recruitment of bacteria during embryogenesis of an earthworm. *ISME J* **2**: 510–518.

Dubilier N, Bergin C, Lott C. (2008). Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. *Nat Rev Microbiol* **6**: 725–740.

Drake HL, Horn MA. (2007). As the worm turns: the earthworm gut as a transient habitat for soil microbial biomes. *Annu Rev Microbiol* **61**: 169–189.

Edwards CA (ed). (2004). *Earthworm Ecology* 2nd edn. CRC Press: Boca Raton, FL, pp 460.

Egert M, Marhan S, Wagner B, Scheu S, Friedrich MW. (2004). Molecular profiling of 16S rRNA genes reveals diet-related differences of microbial communities in soil, gut and casts of *Lumbricus terrestris* L. (Oligochaeta: Lumbricidae). *FEMS Microb Ecol* **48**: 187–197.

Eisenhauer N, Marhan S, Scheu S. (2008). Assessment of anecic behaviour in selected earthworm species: effects on wheat seed burial, seedling establishment, wheat growth and litter incorporation. *Appl Soil Ecol* **38**: 79–82.

Fisher OA, Matlova L, Bartl J, Dvorska L, Svastova P, Du Maine R *et al.* (2003). Earthworms (Oligochaeta, Lumbricidae) and mycobacteria. *Vet Microbiol* **91**: 325–338.

Ihssen J, Horn MA, Matthies C, Gößner A, Schramm A, Drake HL. (2003). N_2O-producing microorganisms in the gut of the earthworm *Aporrectodea caliginosa* are indicative of ingested soil bacteria. *Appl Environ Microbiol* **69**: 1655–1661.

Jolly JM, Lappin Scot HM, Anderson JM, Clegg CD. (1993). Scanning electron microscopy of the gut microflora of two earthworms: *Lumbricus terrestris* and *Octolasion cyaneum*. *Microb Ecol* **26**: 235–245.

Jones CG, Lawton JH, Shachak M. (1994). Organisms as ecosystem engineers. *Oikos* **69**: 373–386.

Killham K, Prosser JI. (2007). The Prokaryotes. In: Paul EA (ed). *Soil Microbiology, Ecology and Biochemistry* 3rd edn. Academic Press: Canada, pp 119–143.

Kirschner RA, Parker BC, Falkinham JO. (1999). Humic and fulvic acids stimulate the growth of *Mycobacterium avium*. *FEMS Microbiol Ecol* **30**: 327–332.

Knapp BA, Podmirseg SM, Seeber J, Meyer E, Insam H. (2009). Diet-related composition of the gut microbiota of *Lumbricus rubellus* as revealed by a molecular fingerprinting technique and cloning. *Soil Biol Biochem* **41**: 2299–2307.

Knapp BA, Seeber J, Podmirseg SM, Meyer E, Insam H. (2008). Application of denaturing gradient gel electrophoresis for analysing the gut microflora of *Lumbricus rubellus* Hoffmeister under different feeding conditions. *Bull Entomol Res* **98**: 271–279.

Kruskal JB. (1964). Multidimensional scaling by optimizing goodness of fit to a nonmetric hypothesis. *Psychometrika* **29**: 1–27.

Ladygina N, Johansson T, Canbäck B, Tunlid A, Hedlund K. (2009). Diversity of bacteria associated with grassland soil nematodes of different feeding groups. *FEMS Microbiol Ecol* **69**: 53–61.

Lattaud C, Locati S, Mora P, Rouland C, Lavelle P. (1998). The diversity of digestive systems in tropical geophagous earthworms. *Appl Soil Ecol* **9**: 189–195.

Lavelle P, Charpentier F, Villenave C, Rossi JP, Derouard L, Pashanasi B *et al.* (2004). Effects of earthworms on soil organic matter and nutrient dynamics at a landscape scale over decades. In: Edwards CA (ed). *Earthworm Ecology*. CRC Press LLC: Boca Raton, FL, pp 145–160.

Lavelle P, Spain AV. (2001). *Soil Ecology*. Kluwer Academic Publishers: Dordrecht, pp 654.

Lee KE.. (1985). *Earthworms, Their Ecology and Relationship with Soils and Land Use*. Academic Press: Sydney, pp 411.

Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS *et al.* (2008). Evolution of mammals and their gut microbes. *Science* **320**: 1647–1651.

Lovley DR, Giovannoni SJ, White DC, Champine JE, Phillips EJ, Gorby YA *et al.* (1993). *Geobacter metallireducens* gen. nov. sp. nov., a microorganism capable of coupling the complete oxidation of organic compounds to the reduction of iron and other metals. *Arch Microbiol* **159**: 336–344.

Moran NA. (2006). Symbiosis. *Curr Biol* **16**: R867–R871.

Odum EP. (1971). *Fundamentals of Ecology* 3rd edn. W.B. Saunders: Philadelphia, pp 574.

Perel TS. (1977). Soil Organisms as Components of Ecosystems: Differences in Lumbricid Organization Connected with Ecological Properties. In: Lohm U, Persson T (eds). *Ecol Bull* **25**: 56–63.

Ritz K, McNicol JW, Nunan N, Grayston S, Millard P, Atkinson D *et al.* (2004). Spatial structure in soil chemical and microbiological properties in an upland grassland. *FEMS Microbiol Ecol* **49**: 191–205.

Sampedro L, Whalen JK. (2007). Changes in the fatty acid profiles through the digestive tract of the earthworm *Lumbricus terrestris* L. *Appl Soil Ecol* **35**: 226–236.

Schmidt O, Curry JP, Dykmans J, Rota E, Scrimgeour CM. (2004). Dual stable isotope analysis ($\delta^{13}C$ and $\delta^{15}N$) of soil invertebrates and their food sources. *Pedobiologia* **48**: 171–180.

Schmidt O, Scrimgeour CM, Handley LL. (1997). Natural abundance of ^{15}N and ^{13}C in earthworms from a wheat and a what-clover field. *Soil Biol Biochem* **29**: 1301–1308.

Schramm A, Davidson SK, Dodsworth JA, Drake HL, Stahl DA, Dubilier N. (2003). *Acidovorax*-like symbionts in the nephridia of earthworms. *Environ Microbiol* **5**: 804–809.

Sims RW, Gerard BM. (1999). *Earthworms: Notes for the Identification of British Species* Synopses of the British Fauna N.S. no. 31, revised. Field Studies Council, Shrewsbury: UK, pp 169.

Singleton DR, Hendrix PF, Coleman DC, Whitman WB. (2003). Identification of uncultured bacteria tightly associated with the intestine of the earthworm *Lumbricus rubellus* (Lumbricidae; Oligochaeta). *Soil Biol Biochem* **35**: 1547–1555.

Thakuria D, Schmidt O, Liliensiek A, Egan D, Doohan FM. (2009). Field preservation and DNA extraction methods for intestinal microbial diversity analysis in earthworms. *J Microbiol Methods* **76**: 226–233.

Thakuria D, Schmidt O, Mac Siúrtáin M, Egan D, Doohan FM. (2008). Importance of DNA quality in comparative soil microbial community structure analyses. *Soil Biol Biochem* **40**: 1390–1403.

Valle-Molinares R, Borges S, Rios-Velazquez C. (2007). Characterisation of possible symbionts in *Onychochaeta borincana* (Annelida: Glossoscolecidae). *Eur J Soil Biol* **43**: S14–S18.

Verma K, Agrawal N, Farooq M, Misra RB, Hans RK. (2006). Endosulfan degradation by a *Rhodococcus* strain isolated from earthworm gut. *Ecotoxicol Environ Safety* **64**: 377–381.

Wan JHC, Wong MH. (2004). Effects of earthworm activity and P-solubilizing bacteria on P availability in soil. *J Plant Nutr Soil Sci* **167**: 209–213.

Wu KS. (1939). On the physiology and pharmacology of the earthworm gut. *J Exp Biol* **16**: 184–197.

Zientz E, Dandekar T, Gross R.. (2004). Metabolic interdependence of obligate intracellular bacteria and their insect hosts. *Microbiol Mol Biol Rev* **68**: 745–770.

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