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Research Article

RESEARCH ARTICLE

Feeding on microbiomes: effects of detritivory on the taxonomic and phylogenetic bacterial composition of animal manures

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One sentence summary: Earthworms build up their gut microbiome by selecting from the pool of ingested bacteria. The core gut microbiome comprised only the 2.6% of total OTUs. Thus, most of the gut microbiome of earthworms is transient.

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ABSTRACT

Earthworms play a key role in nutrient cycling by interacting with microorganisms thus accelerating organic matter turnover in soil systems. As detritivores, some earthworm types ingest and digest a mixture of dead organic matter and microorganisms, like animal manures (i.e. animal gut microbiomes). Here we described the earthworm cast microbiome and the role ingested bacteria play on its composition. We fed *Eisenia andrei* with cow, horse and pig manures and determined the taxonomic and phylogenetic composition of the these manures before and after passage through the earthworm gut. Earthworm cast microbiomes showed a smaller diversity than the manure they fed on. Manures strongly differed in their taxonomic and phylogenetic composition, but these differences were markedly reduced once transformed into earthworm cast microbiomes after passage through the earthworm gut. The core earthworm cast microbiome comprised 30 OTUs (2.6% of OTUs from cast samples), of which 10 are possibly native to the earthworm gut. Most of the core cast microbiome OTUs belonged to phyla *Actinobacteria* and *Proteobacteria*, as opposed to already described animal core gut microbiomes, which are composed mainly of *Firmicutes* and *Bacteroidetes*. Our results suggest that earthworms build up their cast microbiome by selecting from the pool of ingested bacteria.

Key words: bacterial communities; bacterial diversity; Bar-coded pyrosequencing; *Eisenia andrei*; earthworm; core microbiome

INTRODUCTION

Gut microbiomes, the consortia of microorganisms that inhabit the animal gut, are highly specialized microbial communities (Derrien and Van Hylckama Vlieg 2015). Diet is one of the factors that strongly affect the composition of gut microbiomes (Ley *et al.* 2008a,b; Swanson *et al.* 2011; Wu *et al.* 2011; Huttenhower *et al.* 2012; Bolnick *et al.* 2013; Boucias *et al.* 2013; Koeth *et al.* 2013; David *et al.* 2014; Ni *et al.* 2014; Pérez-Cobas *et al.* 2015). Despite vast diet-related differences in the composition

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of the gut microbiome between individuals, there is compelling evidence for the existence of a core gut microbiome in animals (Fraune and Bosch 2007; Turnbaugh *et al*. 2009; Koch and Schmid-Hempel 2011; Roeselers *et al*. 2011; Sekelja *et al*. 2011). The wildly diverse array of microorganisms included in animal gut microbiomes is constantly entering the nutrient cycle when animals egest their faeces. In nature, animal faeces are rapidly decomposed and constitute not only a source of nutrients but also of microorganisms. Soil bacteria and fungi produce the enzymes that cause the biochemical decomposition of organic matter, but detritivorous organisms like earthworms are crucial drivers of the process. Earthworms are involved in the indirect stimulation of microbial populations through fragmentation and ingestion of dead organic matter, which results in a greater surface area for microbial colonization, drastically altering biological activity (Domínguez, Aira and Gómez-Brandón 2010).

Earthworms represent the largest animal biomass component of soil in most temperate terrestrial ecosystems (Lavelle and Spain 2001). They modify microbial biomass and activity and also the microbial community composition in soils (Héry *et al*. 2008; Bernard *et al*. 2012). The main alterations of the microbial communities occur during transit through the earthworm gut, where some microorganisms are digested while others survive and even flourish (Drake and Horn 2007). The earthworm gut has been described as an anaerobic reactor thriving in an aerobic environment with stable conditions of moisture and different nutrient pools (Karsten and Drake 1995, 1997; Horn *et al*. 2005; Drake and Horn 2007). The earthworm gut may therefore act as a biological filter for ingested microbial communities, selecting and/or favouring specific groups of microorganisms (Drake and Horn 2007; Wüst, Horn and Drake 2011). Changes in microbial communities during gut transit are important because the microorganisms are released again to the environment in earthworm casts. Indeed, inoculation of not-processed materials with earthworm casts modifies the rate of organic matter decomposition in the same way as if earthworms were present (Aira and Domínguez 2011).

Earthworms are ecologically classified into three general ecotypes based on their feeding habits (Bouché 1977; Lee 1985): epigeic, endogeic and anecic earthworms, which feed preferentially on dead organic matter, mineral soil and mixtures of plant litter, soil and organic residues, respectively. Although epigeic earthworms are known to play a role in the first steps of the decomposition of organic matter by accelerating degradation rates and nutrient turnover (Aira *et al*. 2008; Gómez-Brandón *et al*. 2010), little is known about how they impact microbial communities (Gómez-Brandón *et al*. 2011a,b; Gómez-Brandón, Lores and Domínguez 2012). Epigeic earthworms are microbivorous and therefore ingest substrates with massive microbial loads. For example, when earthworms feed on animal faeces, they ingest already digested material and also the corresponding gut microbiomes. Thus, earthworms can make use of processed nutrients and, more importantly, of microorganisms as sources of energy (Sampedro, Jeannotte and Whalen 2006; Sampedro and Whalen 2007). There are contradictory results regarding the effect of diet on the composition of gut microbial communities. For example, it seems that for anecic (*Lumbricus terrestris, L. friendi, Aporrectodea longa*) and endogeic (*A. caliginosa*) earthworm species diet modulates the gut microbiome (Tiunov and Scheu 2000; Egert *et al*. 2004; Thakuria *et al*. 2009; Nechitaylo *et al*. 2010). However, in epigeic species diet either may (e.g. *L. rubellus*; Knapp *et al*. 2009) or may not (e.g. *Eisenia andrei*; Gómez-Brandón *et al*. 2011b) shape the earthworm gut microbiome. It is therefore necessary to study how the earthworm gut microbiome is structured. Furthermore, it is unknown whether earthworms possess a core gut microbiome, although a recent study, which extracted DNA from the whole earthworm, has revealed the existence of an earthworm microbiome in the earthworm species *L. rubellus* (Pass *et al*. 2015). This microbiome is mainly comprised of *Proteobacteria* and *Actinobacteria* with minor contributions from phyla *Bacteroidetes* and *Acidobacteria*. We aimed to describe the earthworm gut microbiome and whether the composition of ingested microbiomes from animal faeces (i.e. manures) impact the composition of the earthworm gut microbiome. For this purpose, we applied 16S rRNA pyrosequencing and metagenomic analysis to characterize the taxonomic and phylogenetic composition of bacterial communities in three different types of animal microbiomes, herbivore: (horse), ruminant (cow) and omnivore (pig), that have divergent bacterial communities (Ley *et al*. 2008a,b) before and after passing through the gut of the detritivorous earthworm *E. andrei*. We used *E. andrei* because it is one of the most common epigeic earthworms found in rich organic matter (e.g. litter mounds and herbivore dung) and anthropogenic environments (e.g. manure heaps, vegetal debris and vermicomposting beds) in agricultural landscapes (Domínguez, Aira and Gómez-Brandón 2010).

MATERIALS AND METHODS

Animal manure, earthworms and cast sampling

Five specimens of *E. andrei* were obtained and hand sorted from each of three stock cultures fed exclusively with horse, cow or pig manure for more than five years. The three stock cultures were established with earthworms sampled from one population. The different types of animal manures were collected from farms near the University of Vigo (Galicia, NW Spain) and stored under laboratory conditions (20°C) for maintenance of stock cultures and to be used in the present study.

Earthworms were placed in separate sterile plastic Petri dishes that were partly filled (75%) with vermicompost from each stock culture. Vermicompost is comprised of casts (Domínguez, Aira and Gómez-Brandón 2010), so although earhworms do not feed on their faeces, any accidental ingestion would result in the incorporation of the same bacteria pool already present in the casts. The earthworms were fed *ad libitum* with the same type of animal manure as in the stock culture (breeding dishes). The manure added was taken from the stored manure. Dishes were stored in random positions in an incubation chamber (20°C and 90% relative humidity). To obtain cast samples, earthworms were removed from the dishes, washed three times with sterile distilled water and placed in clean, sterile Petri dishes on moistened sterile filter paper (sampling dishes). All handling was done under sterile conditions in a laminar flow cabinet. Sampling dishes were then placed in the same incubation chamber for 24 h. Earthworms were then returned to the breeding dishes and casts were collected from each sampling dish with a sterile spatula (sterilized between samplings). Casts were then stored in 1.5 mL Eppendorf tubes at –80°C. This process was also done under sterile conditions and repeated (at least five times) to yield 0.25 g of fresh cast material per earthworm and manure.

DNA extraction and bar-coded pyrosequencing

DNA from cast samples and each type of manure (0.25 g) was extracted using the PowerSoil DNA Isolation kit (MoBio Laboratories Inc., Carlsbad, California), according to the

manufacturer's protocol. A fragment of the 16S rRNA gene covering the V2 (forward 5′-AGYGGCGIACGGGTGAGTAA) and V3 (reverse 5′-ATTACCGCGGCTGCTGG) regions was amplified using primers and a touchdown PCR protocol described by Sundquist *et al.* (2007). The primers were modified from Sundquist *et al.* (2007) and designed to include (5′ to 3′) the 21 bp Titanium 454 primer A, the 4 bp key, and the V2 (forward) sequence for the forward primer. The reverse primer was similarly constructed and included the Titanium 454 primer B, the 4 bp key followed by a 10 bp DNA Barcode (MID: Roche Technical Bulletin No. 005–2009) and the V3 (reverse) sequence. For PCR amplification, we used AccuPrimeTM Pfx DNA Polymerase from Invitrogen in a single 14 μl reaction (1.25 μl 10× buffer, 8.5 μl H20, 0.25 μl Taq, 1 μl each of 2.5 μM forward and reverse primer and 2 μl of gDNA). Following successful amplification, samples were shipped to the sequencing center at Brigham Young University, Provo, UT (USA) for 454 Titanium pyrosequencing. Primer dimers were eliminated using AMPure beads. Libraries were pooled in equal amounts according to the total quantity of DNA (as estimated with Quant-iT PicoGreen) and then sequenced using a Roche 454 sequencer.

Processing of pyrosequencing data

Data from raw standard flowgram format (sff) files were processed with mothur (version 1.34.2, Schloss *et al.* 2009). We used default settings to minimize the sequencing error as described by Schloss, Gevers and Westcott (2011). Briefly, sequence reads were separated according to their primer and barcode and denoised. Sequence reads were then trimmed to remove barcode and primer sequences and yield sequences with a minimum of 200 bp. Clean reads were aligned to the bacterial-subset SILVA alignment, provided at http://www.mothur.org. Sequences were screened to cover the same genetic space and filtered to remove columns without alignment data. Finally, sequences were pre-clustered to remove those including pyrosequencing errors. Chimeras were checked with the chimera.uchime command in mothur and then removed. Sequences were classified with the naïve Bayesian classifier (Wang *et al.* 2007) included in mothur, and any contaminants (sequences classified as mitochondria, chloroplast, archaea, eukaryote or not classified) were removed. To obtain operational taxonomic units (OTUs) at the 0.03 level, we first constructed a distance matrix (cut-off 0.15) and then clustered the resulting sequences into OTUs and classified them to obtain their consensus taxonomy. Sequence data (raw sff files) have been uploaded to the GenBank SRA database under accession number SRA245228.

Statistical analysis

Samples were subsampled to the smallest sample size (974 sequences) to remove the effect of sample size bias on community composition; this yielded a total of 21 samples: 3 replicates for each of the three manures and 5, 4 and 3 replicates for casts from earthworms fed with horse, cow and pig manure, respectively. We defined the core microbiome of the *E. andrei* casts as that comprised of OTUs present in at least 10 of the 12 samples of casts. We used the minimum entropy decomposition (MED) approach in order to identify whether the association of core OTUs with earthworms is strain specific, or whether those from the three diets could harbour slightly different microbiotas. MED analysis, which expands the oligotyping analysis to a whole data set instead of to specific taxa, enables sensitive discrimination of closely related organisms in marker gene amplicon datasets with a resolution of one nucleotide (Eren *et al.* 2015). To do this, we took all sequences related to OTUs from the core microbiome, aligned them with Pynast against the 13.8 version of Greengenes database and trimmed the alignment to the same number of base pairs before running the MED analysis (Eren *et al.* 2015).

An approximately maximum-likelihood phylogenetic tree was inferred using FastTree 2.1 (Price, Dehal and Arkin 2010). Taxonomic alpha-diversity was calculated as the number of observed OTUs (Sobs) and as estimated diversity and richness (Shannon and Chao1 indexes, respectively). Phylogenetic diversity was calculated as Faith's phylogenetic diversity (Faith 1992). The effect of type of manure (horse, cow and pig) and earthworm gut transit (before and after) on both taxonomic and phylogenetic alpha-diversity of bacterial communities from manure and casts was analysed by a two-way ANOVA of linear models, with type of manure (pig, horse and cow) and gut transit (before and after) as fixed factors. The normality of residuals and homogeneity of variance across groups was checked for each variable. Tukey's test was used for post-hoc comparisons and Benjamini–Hochberg FDR was used as multiple test correction method (library multcomp; Hothorn, Bretz and Westfall 2008).

Taxonomic beta-diversity at the OTU level was estimated as the difference in bacterial taxonomic community composition between cast and manure samples. This was done by coupling principal coordinate analysis (PCoA) with distance matrixes that take the abundance of OTUs into account (Bray–Curtis) or not (Jaccard). Phylogenetic beta-diversity was also estimated by PCoA of weighted (considering abundance of OTUs) and unweighted unifrac matrix distances (Lozupone and Knight 2005) obtained as average values after sampling the phylogenetic tree 1000 times. All PCoAs were carried out using the phyloseq library (McMurdie and Holmes 2013) with BIOM files and distance matrixes obtained via mothur. Permutational multivariate analysis tests (PERMANOVA, adonis function with 10000 permutations, from library vegan, Oksanen *et al.* 2015) were used to compare taxonomic and phylogenetic community composition. All analyses were performed with R 3.1 (2014) and mothur (version 1.34.2; Schloss *et al.* 2009).

RESULTS

Bacterial composition changes of manures after transit through the earthworm gut

After raw file processing, 20 454 sequences were obtained, resulting in 1813 OTUs (defined at 97% sequence similarity) after rarefaction to lowest sample size. Rarefaction curves indicated that the sampling depth was more optimal for casts than for manure samples (Fig. S1, Supporting Information). The composition of bacterial communities of the three types of manures was strongly affected by passing through the gut of *E. andrei*, and the effect was evident at phylum (Fig. 1; Fig. S2, Supporting Information) and OTU level (Fig. 3). Thus, some phyla (e.g. *Actinobacteria*) were more abundant in casts than in manure, whereas others (e.g. *Bacteroidetes* and *Spirochaetes*) decreased in abundance after gut transit (Fig. 1). Changes in abundance depended on phylum and type of manure. Hence, the abundance of *Proteobacteria* decreased and increased in casts produced by worms fed pig (before: 60%, after: 32%) and cow manure (before: 24%, after: 82%) (interaction type of manure and gut transit, $P < 0.001$). Within *Proteobacteria*, we found that transit through the gut increased the abundance of *Gammaproteobacteria* (before: 43%, after: 54%; gut transit, $P < 0.0001$), the most abundant class, but only significantly in cow manure (before: 21%,

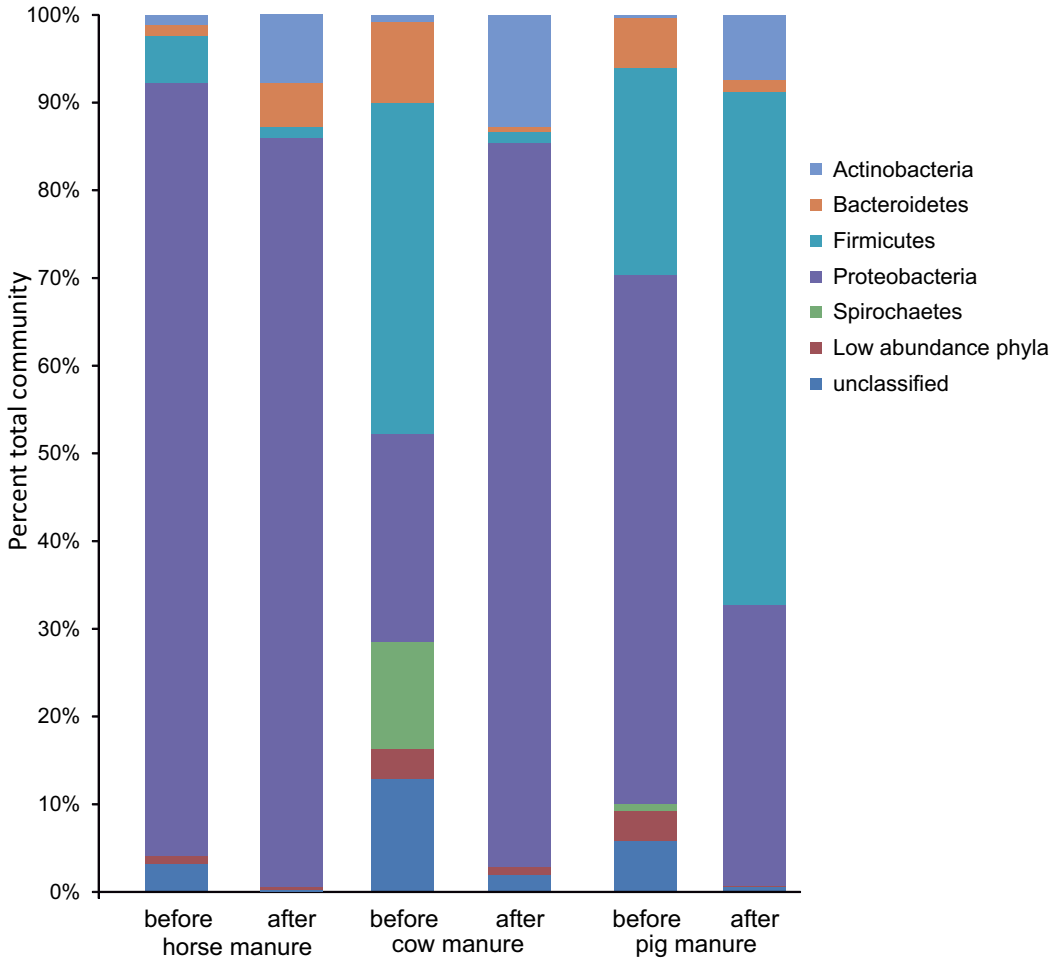

Figure 1. Bacterial community composition (phylum level) of animal manures (horse, cow or pig manure) before and after being digested by the earthworm *E. andrei*. Low abundance bacterial phyla (<3%) were grouped (*Acidobacteria*, *Candidatus_Saccharibacteria*, *Chloroflexi*, *Cloacimonetes*, *Fibrobacteres*, *Hydrogenedentes*, *Ignavibacteria*, *Nitrospirae*, *Planctomycetes*, *Synergistetes*, *Tenericutes* and *Verrucomicrobia*).

after: 56%; Fig. S3, Supporting Information). In *Betaproteobacteria*, *Deltaproteobacteria* and *Epsiloproteobacteria* their abundances after transit through the gut varied with on manure type. Thus, gut transit significantly reduced the abundance of *Betaproteobacteria* in horse (before: 5.7%, after: 0.2%) and pig manure (before: 15%, after: 0.03%) only (interaction, $P < 0.001$), whereas in *Deltaproteobacteria* (interaction, $P < 0.001$) and *Epsilonproteobacteria* ($P = 0.014$) it decreased their abundances only in pig (before: 8%, after: 0%) and cow manure, respectively (before: 1%, after: 0%; Fig. S3, Supporting Information). We did not find any change in the abundance of *Alphaproteobacteria* after transit through the gut. In addition, passage of animal manure through the earthworm gut increased the abundance of *Acidobacteria* in casts from worms fed cow manure (before: 0%, after: 0.3%) and decreased it in cast from worms fed pig manure (before: 0.25%, after: 0.01%) (interaction, $P = 0.024$; Fig. S2, Supporting Information). We found a reverse trend for *Firmicutes*, with an increase in casts from pig (before: 24%, after: 59%) and a decrease in casts from cow manure (before: 38%, after: 1%) (interaction, $P < 0.001$, Fig. 1). This was also the case for *Bacteroidetes*, with a significant decrease of abundance only in cow manure (before: 9%, after: 0.5%) (interaction, $P < 0.001$, Fig. 1). In *Actinobacteria*, gut transit increased its abundance independently of manure type (before: 0.7%, after: 9.2%) (gut transit, $P = 0.002$). *Hydrogenedentes* (before: 0.01%, after: 0%) and *Chloroflexi* (before: 0.9%, after: 0.1%) decreased in abundance after transit through the gut only in casts of worms fed pig manure (interaction, $P = 0.021$ and $P < 0.001$, respectively, Fig. S2, Supporting Information), whereas *Synergistetes* (before: 0.1%, after: 0%) and *Spirochaetes* (before: 12%, after: 0%) were not present in casts from worms fed with manure (interaction, $P < 0.001$ for both, Fig. S2, Supporting Information), and *Tenericutes* (before: 0.04%, after: 0%) did not appear in casts from worms fed horse manure (interaction, $P = 0.027$, Fig. S2, Supporting Information).

Alpha-diversity of bacterial communities of the three types of manures decreased markedly after transit through the gut of *E. andrei*. The number of observed OTUs decreased depending on manure type (range: 30–278; ANOVA interaction manure × gut transit, $F_{2,15} = 8.68$, $P = 0.003$), as well as it occurred with phylogenetic diversity (Faith PD range: 14.98–59.86; ANOVA, $F_{2,15} = 3.90$, $P = 0.043$). Estimated taxonomic richness (Chao1 range: 82–695; ANOVA, $F_{1,15} = 160.85$, $P < 0.0001$) and taxonomic diversity decreased after transit through the gut (Shannon range: 0.94–4.71; ANOVA, $F_{1,15} = 76.78$, $P < 0.0001$). In all cases, the post-hoc tests revealed significant differences for each manure–cast combination (Fig. 2). Animal manure transit through the gut of *E. andrei* changed the taxonomic composition of the bacterial

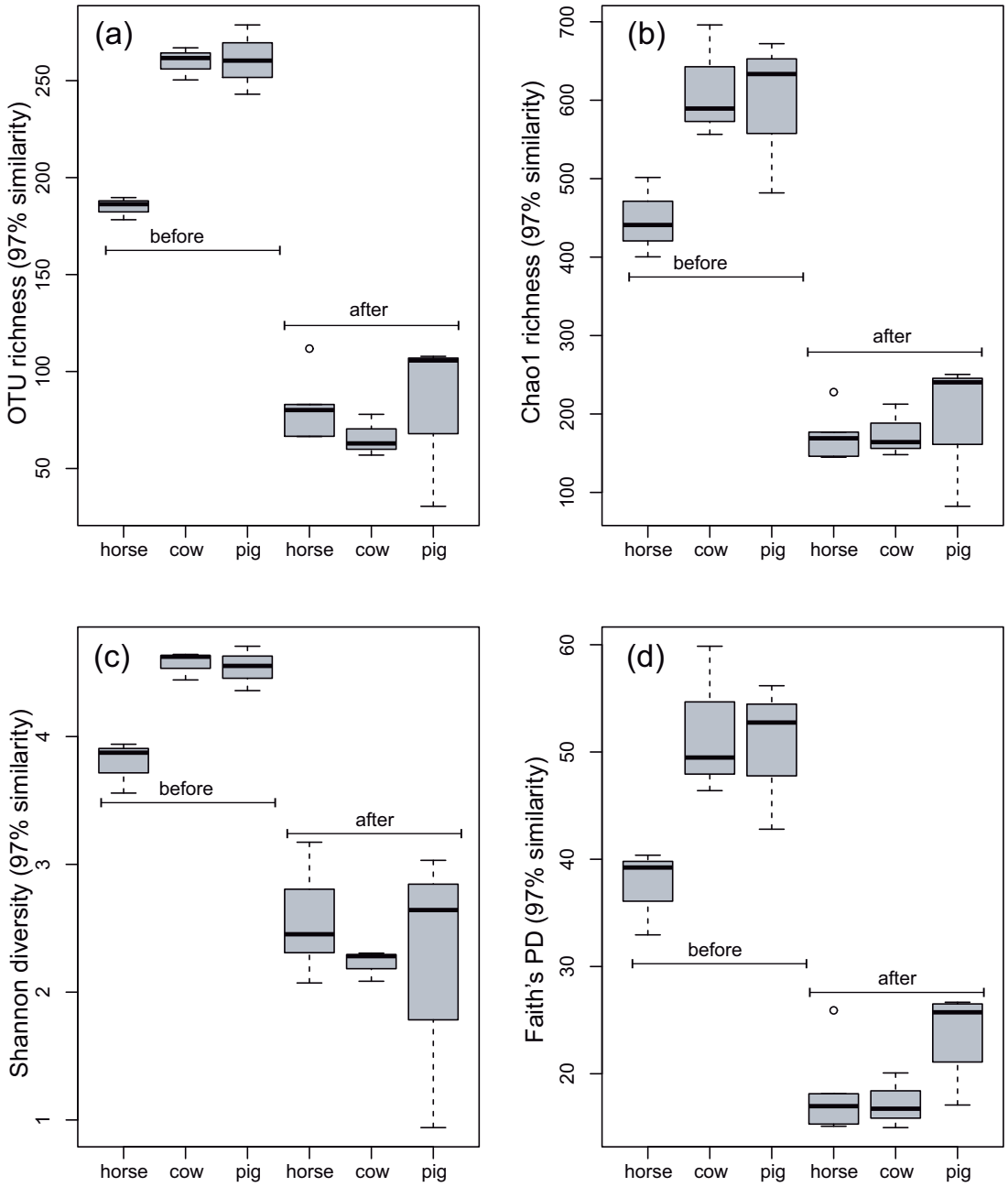

Figure 2. Diversity of animal microbiomes (horse, cow and pig) before and after being digested by the earthworm *E. andrei*. (**a**) Observed OTU richness, (**b**) estimated taxonomic richness (Chao 1), (**c**) taxonomic diversity (Shannon index) and (**d**) phylogenetic diversity (Faith's PD).

community for all manure types. The type of manure and transit though the gut affected both the presence-absence (Fig. 3) and abundance of OTUs indexes (Fig. S4a, Supporting Information). The same pattern emerged for the phylogenetic composition of the bacterial community, with differences between casts and each type of manure in both unweighted Unifrac (Fig. 3b) and weighted Unifrac (Fig. S4b, Supporting Information) distances. These interactions demonstrate that the gut of *E. andrei* acted as a filter by reducing the variation between the ingested bacterial communities.

The core cast microbiome of *E. andrei*

The core cast microbiome of the earthworm *E. andrei* comprises 30 OTUs from a total of 1145 OTUs present in cast samples (2.6%) (Table 1). The OTUs included were some of the most abundant ones (OTUs, 0.05–17.52%, Table 1) and mainly belonged to the phyla *Proteobacteria* (43%), *Actinobacteria* (40%) and *Firmicutes* (14%), with minor contributions from *Bacteroidetes* (3%). Within these phyla, the most abundant OTUs were those classified as *Acinetobacter*, *Pseudomonas*, *Clostridium_sensu_stricto_*

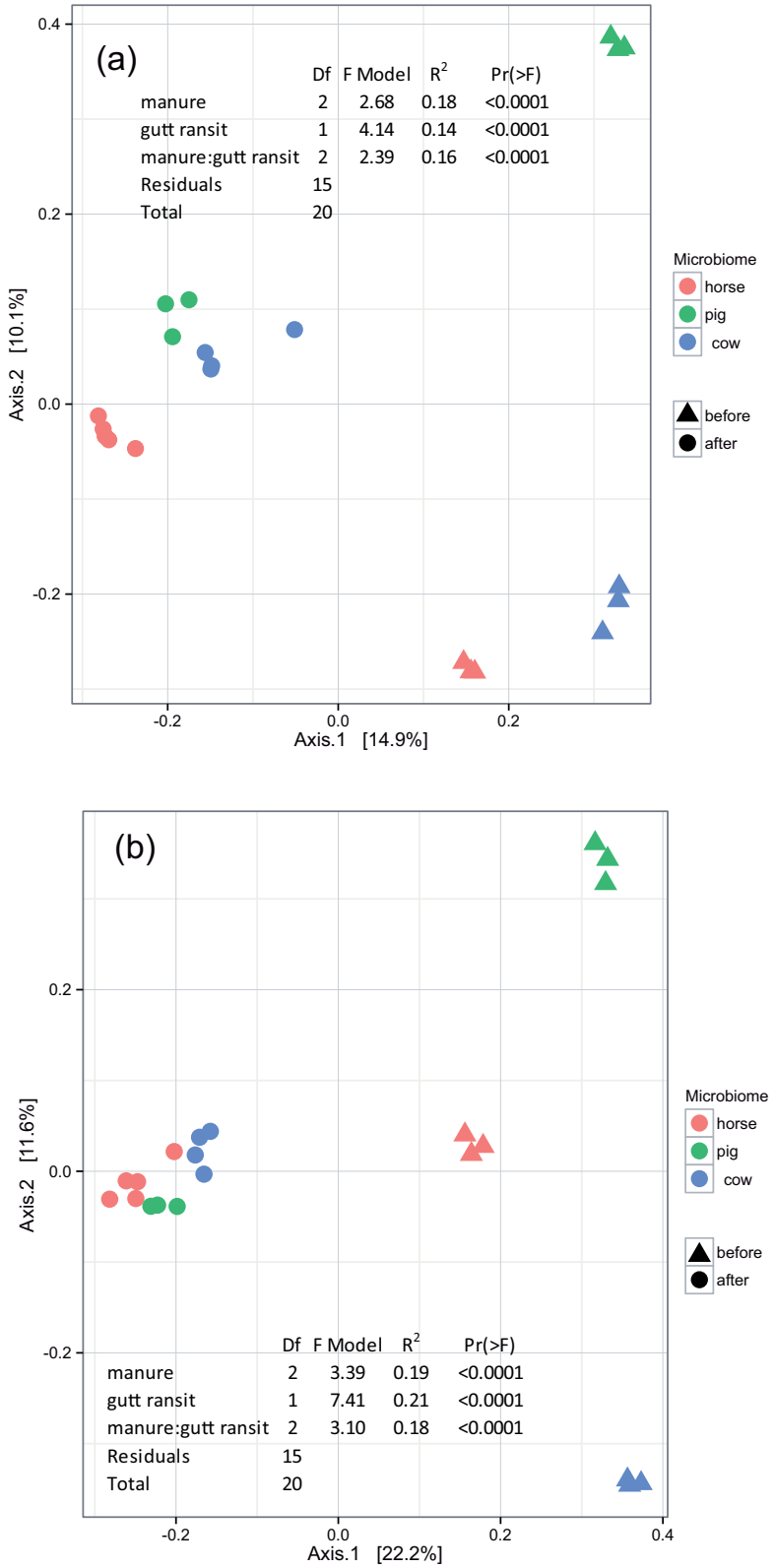

Figure 3. PCoA of (**a**) taxonomic (Jaccard), and (**b**) phylogenetic (unweighted UniFrac) β-diversity of animal microbiomes (pig, horse and cow) before and after being digested by the earthworm *E. andrei*.

Table 1. Taxonomy (phylum and genus) of OTUs forming the core bacterial community of the gut of *E. andrei*. We defined the core microbiome of the *E. andrei* casts as that comprised of OTUs present in at least 10 of the 12 samples of casts. Native OTUs, i.e. those that were present only in samples of earthworm casts, are marked in bold.

OTU	Percentage	Taxonomy
Otu0001	17.52	Proteobacteria, *Acinetobacter*
Otu0002	7.83	Proteobacteria, *Pseudomonas*
Otu0003	7.62	Proteobacteria, *Acinetobacter*
Otu0004	6.69	Firmicutes, *Clostridium_sensu_stricto*
Otu0005	3.40	Proteobacteria, *Acinetobacter*
Otu0006	3.20	Firmicutes, *Clostridium_XI*
Otu0007	2.82	Firmicutes, *Clostridium_sensu_stricto*
Otu0008	2.74	Proteobacteria, *Amaricoccus*
Otu0009	2.61	Proteobacteria, *Acinetobacter*
Otu0011	1.59	Proteobacteria, *Acinetobacter*
Otu0012	1.41	Actinobacteria, *Rhodococcus*
Otu0018	**0.83**	**Bacteroidetes, *Chryseobacterium***
Otu0019	0.67	Proteobacteria, *Acinetobacter*
Otu0022	0.58	Actinobacteria, *Microbacterium*
Otu0030	0.37	Actinobacteria, *Demequina*
Otu0041	0.24	Actinobacteria, *Nocardioides*
Otu0050	**0.20**	**Actinobacteria, *Nocardioides***
Otu0056	**0.17**	**Actinobacteria, *Mycobacterium***
Otu0063	0.15	Proteobacteria, *Acinetobacter*
Otu0065	0.14	Proteobacteria, *Brevundimonas*
Otu0066	**0.14**	**Proteobacteria, *Rhodobacter***
Otu0071	**0.13**	**Actinobacteria, *Microbacterium***
Otu0072	0.12	Firmicutes, *Caryophanon*
Otu0075	**0.12**	**Actinobacteria, *Angustibacter***
Otu0079	**0.11**	**Actinobacteria, *Kutzneria***
Otu0095	0.10	Proteobacteria, *Rhodobacter*
Otu0107	0.08	Proteobacteria, *Ochrobactrum*
Otu0110	**0.07**	**Actinobacteria, *Mycobacterium***
Otu0124	**0.06**	**Actinobacteria, *Gaiella***
Otu0132	**0.05**	**Actinobacteria, *Aciditerrimonas***

Clostridium_XI, *Clostridium_sensu_stricto*, *Amaricoccus*, *Chryseobacterium*, *Microbacterium* and *Demequina* (Table 1). A total of 10 of these OTUs were not found in the animal manure samples, indicating that they may represent the native bacteria of the *E. andrei* gut. However, subsampling may have affected the presence of those OTUs in manure samples, which showed higher levels of alpha-diversity (Fig. S1, Supporting Information). These native bacteria mainly comprised members of the phylum *Actinobacteria* (80%), classified as *Nocardioides*, *Mycobacterium*, *Microbacterium*, *Angustibacter*, *Gaiella*, *Kutzneria* and *Aciditerrimonas* (Table 1). There also was an OTU from the phylum *Bacteroidetes* (*Chryseobacterium*) and another from the *Proteobacteria* (*Rhodobacter*). A total of 8 of these OTUs were present in 10 samples, whereas the other 2 OTUs were present in 11 and 12 samples, respectively.

In order to get a more detailed analysis of OTUs from the core cast microbiome, we did an MED analysis of the sequences from all the OTUs comprising the core cast microbiome. The MED analysis split OTUs from the core cast microbiome into 135 MED nodes as opposed to the 30 OTUs they came from (an increase of 4.5 times over the number of OTUs). No trend was observed in the distribution of MED nodes among samples of casts, although we found a few nodes present only in cast samples from earthworms fed with horse (10), cow (4) and pig manure (9). There were 81 MED nodes found only in cast samples indicating that they may represent the native bacteria of the *E. andrei* casts. However, only 22 of these 81 were present in at least 10 cast samples. These 22 MED nodes should be regarded as the core cast microbiome. Finally, only 2 (14 and 169) of these 22 MED nodes were present in all cast samples.

DISCUSSION

Changes in bacterial composition of manures after transit through the earthworm gut

Since the publication of the seminal paper by Ley *et al.* (2008a) on the evolution of animal gut microbiomes, studies involving the composition of gut microbiomes have generally revealed that 'we are what we eat', with diet governing the composition in most of the studied gut microbiomes (Ley *et al.* 2008b; Swanson *et al.* 2011; Wu *et al.* 2011; Huttenhower *et al.* 2012; Bolnick *et al.* 2013; Boucias *et al.* 2013; Koeth *et al.* 2013; David *et al.* 2014; Ni *et al.* 2014; Pérez-Cobas *et al.* 2015). We also found that the composition of the cast microbiome of the earthworm *E. andrei* is driven largely by the composition of the ingested manure. Moreover, the analysis of the manures ingested by the earthworms revealed that the gut seems to act as a biological filter for the ingested bacterial pool. The cast microbiomes of *E. andrei* tended to converge at taxonomic and phylogenetic levels, being more similar at both levels than those from the manures. This is an important finding because animal gut microbiomes differed widely depending on whether they were derived from omnivores (pig), herbivores (horse) or ruminants (cow). Our data support the idea that bacteria are selectively screened in the earthworm gut during digestion (Schönholzer, Hahn and Zeyer 1999; Schönholzer *et al.* 2002; Sampedro, Jeannotte and Whalen 2006; Sampedro and Whalen 2007; Gómez-Brandón *et al.* 2011b; Gómez-Brandón, Lores and Domínguez 2012), thus resulting in the massive reduction of bacterial diversity that we found. This decrease of bacterial diversity in the cast microbiome with respect to the ingested manures was also observed in the whole microbiome of *L. rubellus* with respect to surrounding soil environment. Consequently, members of the phyla *Tenericutes*, *Synergistetes* and *Spirochaetes* did not appear in the earthworm cast microbiome, whereas they appeared in the initial manures.

Our findings regarding how the composition of manures modulate the cast microbiome of *E. andrei* are consistent with those reported for other earthworm species. Previous studies using lower resolution technics have shown that earthworm gut and/or cast microbiomes of the species *A. caliginosa, A. longa*, *L. terrestris*, *L. friendi* and *L. rubellus* are a subset of those soil microbiomes where they live (Egert *et al.* 2004; Knapp *et al.* 2009; Thakuria *et al.* 2009). Moreover, changes in the soil microbiome and/or organic matter added to soils lead to changes in earthworm gut and/or cast microbiomes (Egert *et al.* 2004; Knapp *et al.* 2009; Thakuria *et al.* 2009; present study). Indeed, data from these studies show interactions between soil and earthworm gut and/or cast microbiomes similar to those reported in the present study.

The composition, at phylum level, of the cast microbiome of *E. andrei* is relatively similar to that of other epigeic earthworm species such as *E. fetida* and *L. rubellus* (Furlong *et al.* 2002; Singleton *et al.* 2003; Knapp *et al.* 2009; Byzov *et al.* 2009), and to that of the anecic earthworm *L. terrestris* (Wüst, Horn and Drake 2011). Thus, bacterial communities from the guts and/or casts of these earthworm species are dominated by variable amounts of members of the phyla *Proteobacteria*, *Firmicutes*, *Bacteroides* and *Actinobacteria* depending on the diet of earthworms. Other

bacterial phyla with a minor representation in the gut and/or casts of these earthworm species are *Verrucomicrobia*, *Acidobacteria* and *Chloroflexi*.

The core microbiome of the earthworm *E. andrei*

Despite the important role that manures play in shaping the composition of the earthworm cast microbiome, our data also show the existence of a core cast microbiome in the earthworm *E. andrei*; although this core accounts for only 2.6% of the OTUs present in the earthworm cast microbiome. This indicates that most of the cast microbiome of *E. andrei* is transient and heavily dependent on the bacterial communities that they ingest. To our knowledge, this is the first description of a core cast microbiome in an earthworm species. A total of 10 of these OTUs were not derived from the manures, i.e. they were only present in the earthworm cast microbiome, thus being potential native bacteria in the gut of *E. andrei*. We chose the term native instead of symbiont because native OTUs should be present in all casts samples to be considered symbionts. Hence, only OTU0110 (two MED nodes in all samples), classified as *Mycobacterium*, could be considered a symbiont of the *E. andrei* gut (Table 1). A deep examination with MED analysis of the sequences from OTUs belonging to the core microbiome showed 135 MED nodes. However, the core microbiome dropped from 30 OTUs to 22 MED nodes. This reduction was due to the sensitivity of MED analysis, which detects variations in one nucleotide (Eren *et al*. 2015); this increase in bacterial diversity hinders the possibility for an MED node to appear in several samples. Thus, MED analysis revealed that different manures resulted in slightly different earthworm microbiomes, because most of the MED nodes did not show any unique association with samples coming from horse, cow or pig manures. Moreover, the presence of OTUs that did not appear in any manure suggests that these bacteria may be transferred vertically, as occurs with nephridial bacterial symbionts found in earthworms (Davidson and Stahl 2006, 2008; Davidson, Powell and Stahl 2010), and may be subjected to codiversification. It is important to highlight that our experiments were carried out with laboratory stocks of *E. andrei*, and it is possible that the cast microbiome of wild *E. andrei* is different. However, in nature this earthworm species lives in fresh organic matter in forest litter, litter mounds, herbivore dung and anthropogenic environments common in agricultural landscapes (Domínguez, Aira and Gómez-Brandón 2010). There is a recent study describing the microbiome of *L. rubellus* (Pass *et al*. 2015), which has unveiled the existence of a core microbiome for this earthworm species. However, this study extracted and sequenced DNA from the whole earthworm body, so it is not possible to assign the OTUs found to different biological compartments, which presumably have different bacterial communities [e.g. nephridia (Davidson, Powell and James 2013) versus gut (our study)]. Interestingly, the proportions of OTUs belonging to different bacterial phyla were very similar to those in our study. Pass *et al*. (2015) showed a core whole microbiome comprised of *Proteobacteria* (50%), *Actinobacteria* (30%), *Bacteroidetes* (6%) and *Acidobacteria* (3%), and only lacks *Firmicutes*, which appeared in our study. However, the proportion of OTUs included in the core whole microbiome of *L. rubellus* regarding its whole microbiome is higher (21% identified at genus level and 5.4% of OTUs defined at 97% similarity) than what we have found for the cast microbiome of *E. andrei*. Another important difference between *L. rubellus* and *E. andrei* microbiomes is their composition at higher taxonomic levels (genus level); the *L. rubellus* microbiome is dominated by *Serratia* (72% of the core OTU abundance), while the *E. andrei* microbiome is dominated by members of genus *Acinetobacter* (Table 1). These differences in composition could be both sample related (i.e. whole microbiome versus cast microbiome) and species related, as seen in other animal species (Ley *et al*. 2008a,b). In general, the core cast microbiome of the zebrafish, hoatzin, humans, ascidians, alligator, oyster and several species of termites comprised variable proportions of OTUs from the phyla *Firmicutes* and *Bacteroidetes* (Godoy-Vitorino *et al*. 2008; Turnbaugh *et al*. 2009; Roeselers *et al*. 2011; King *et al*. 2012; Keenan, Summers Engel and Elsey 2013; Otani *et al*. 2014). They are very different from the core cast microbiome of *E. andrei*, which is dominated by members of the phyla *Proteobacteria* and *Actinobacteria*.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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