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Characterization of the bacterial communities of casts from *Eisenia andrei* fed with different substrates

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ABSTRACT

Earthworms play a key role during the first stage of decomposition by enhancing the activity of microorganisms. As organic matter passes throughout the earthworm gut, nutrient pools and microbial communities are modified and released in casts. Here we used 16S rRNA pyrosequencing and metagenomic analysis to characterize the bacterial communities of casts from the earthworm *Eisenia andrei* fed with different food sources (cow, horse and pig manure). We found that the bacterial communities of cast strongly depended on the food source ingested by earthworms; although, no differences in α-diversity were detected. Bacterial communities of casts were mainly comprised of a variable amount of OTUs (operational taxonomic unit) belonging to the phyla Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes, with minor contributions from the phyla Verrucomicrobia, Chloroflexi, Hydrogenedentes, Latescibacteria, Planctomycetes and Candidatus Saccharibacteria. From these bacterial profiles we found OTUs that worked out as biomarkers for each bacterial community allowing us to discriminate among food sources.

1. Introduction

Earthworms are key components of temperate soil ecosystems, where they constitute the largest biomass and contribute to the key process of decomposition. Although the biochemical decomposition of organic matter is primarily accomplished by microorganisms, earthworms are crucial drivers of the process. Earthworms are involved in the stimulation of microbial populations through ingestion and fragmentation of fresh organic matter, which results in a greater surface area available for microbial colonization, thereby drastically altering biological activity (Edwards, 2004; Domínguez et al., 2010). Earthworm–microbe interaction, and the resulting modified microbial communities (Aira et al., 2008; Gómez-Brandón et al., 2011a) enhances rates of decomposition by, for example, increasing the rates of cellulolytic metabolism (Aira et al., 2006), microbial enzymatic activity (Aira et al., 2007a) or microbial metabolic capabilities (Aira et al., 2007b). Changes in the composition of microbial communities during gut transit play a major role in the decomposition process as the modified microbial communities are released to the environment as part of the earthworm casts. In fact, inoculation of raw residues with earthworm casts modifies the rate of organic matter decomposition in the same way as if earthworms were present (Aira and Domínguez, 2011). During transit through the earthworm gut some bacterial groups may be digested and others may survive and even flourish (Drake and Horn, 2007). Hence, it is important to understand how gut transit modifies the bacterial populations ingested by earthworms. Studies investigating the direct effect of earthworms on microorganisms are in need particularly for epigeic earthworm species because most such studies focus on soil-dwelling endogeic and anecic species. In nature, epigeic earthworms live in fresh organic matter of forest litter, in litter mounds, in herbivore dungs, and in anthropogenic environments such as manure heaps, vegetal debris and vermicomposting beds common in agricultural landscapes. There are several studies characterizing the bacterial communities of casts from epigeic earthworm species. Thus, the composition of bacterial communities of casts from *Lumbricus rubellus* seem to depend on ingested bacterial communities (Furlong et al., 2002; Singleton et al., 2003; Knapp et al., 2009) as is the case with endogeic and anecic species (Egert et al., 2004; Thakuria et al., 2009). However, it is not the case for cast of *Eisenia andrei* fed with different diets

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(Gómez-Brandón et al., 2011bKoubová et al., 2015). Nevertheless, these studies have either used PLFAs, DGGE or cloning and sequencing, which due to their intrinsic or applied technical limitations, underestimate bacterial diversity. Thus, our aims were to characterize the taxonomic and phylogenetic composition of the bacterial communities residing in casts from the earthworm *E. andrei* and to ascertain the contribution of ingested bacteria to its bacterial community composition. To do this we used 16S rRNA pyrosequencing and metagenomic analysis of casts from the earthworm *E. andrei* fed with three substrates that heavily differ in their bacterial composition (cow, horse and pig manure; Ley et al., 2008). We also assess whether bacterial communities of casts from different manures constitute unique bacterial communities (i.e., taxonomic biomarkers) or share a variable proportion of their members.

2. Material and methods

2.1. Animal manures, earthworms and casts sampling

Animal manures (horse, cow and pig manure) were collected from a farm near the University of Vigo (Galicia, NW Spain) and stored under laboratory conditions (20 °C). We sampled five specimens of *E. andrei* (hand-sorted method) from different stock cultures that were fed with the three animal manures from at least 7 years. *E. andrei* was selected as earthworm model species as its importance in vermicomposting and because is one of the most common and abundant epigeic earthworms found in natural (e.g., litter mounds and herbivore dungs) and anthropogenic environments (e.g., manure heaps, vegetal debris and vermicomposting facilities) rich in organic matter (Domínguez et al., 2010). The earthworms were placed in separate sterile plastic Petri dishes (one per dish); each dish was filled (75% of space) with vermicompost from each stock culture) and earthworms were fed ad libitum with one of the three animal manures (breeding dishes). The dishes were stored in random positions in an incubation chamber, at 20 °C and 90% relative humidity. In order to obtain cast samples, earthworms were removed from the dishes, washed three times with sterile distilled water and placed in clean and sterile Petri dishes on moistened sterile filter paper (sampling dishes). This was done under sterile conditions in a laminar flow cabinet. Sampling dishes were placed in the same incubation chamber during 24 h. After that, earthworms were returned to the breeding dishes and casts were recovered from each sampling dish with a sterile spatula, which was sterilized between earthworms from the same diet and between diets. Casts were stored in 1.5 mL Eppendorf tubes at −80 °C. This process was done again under sterile conditions and repeated (a minimum of five times) until attaining 0.25 g of fresh casts per earthworm and manure type.

2.2. DNA extraction and bar-coded pyrosequencing

Total DNA from casts (0.25 g) was extracted with the PowerSoil DNA Isolation kit (MoBio Laboratories Inc., Carlsbad, California) according to the manufacturer's protocol. We amplified a fragment of the 16S rRNA gene spanning the V2 (start: 101, end: 361) and V3 (start: 338, end:534) regions by using the primers (forward 5'-AGYGGCGIACGGGTGAGTAA and reverse 5'-ATTACCGCGGCTGCTGG) and touchdown PCR protocol described by Sundquist et al. (2007). Our primers were modified from Sundquist et al. (2007) to include (from 5′–3′) the 21 bp Titanium 454 primer A, the 4 bp key, and the V2 (forward) for our forward primer; while our reverse primer included the Titanium 454 primer B, the 4 bp key, a 10 bp DNA Barcode (MID: Roche Technical Bulletin No. 005-2009) and the V3 (reverse) primer. Using our primers, each sample could proceed directly to pyrosquencing following PCR amplification. We used AccuPrime™ Pfx DNA Polymerase from Invitrogen in a single 14 μl reaction (1.25 μl 10x 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 submitted to the sequencing center at Brigham Young University. They were cleaned of primer dimer using AMPure beads, pooled in equal amounts according to the total quantity of DNA (as estimated with Quant-iT PicoGreen), and sequenced using a Roche 454 sequencer. We submitted to sequencing 5 samples per treatment (horse, cow and pig) but only 5, 4 and 3 samples were successfully sequenced for cast obtained from earthworms fed with horse, cow and pig manure respectively.

2.3. Processing of pyrosequencing data

Data from raw standard flowgram format (sff) files were processed with mothur (version 1.35.1, Schloss et al., 2009). The default settings were used to minimize the sequencing error described by Schloss et al. (2011). Briefly, the flow grams were separated according to their primer and barcode sequence, and the sequence data were de-noised. The sequence reads were first trimmed to remove barcode and primer sequences. Only sequences ≥200 bp were aligned to the bacterial-subset SILVA alignment available at http://www.mothur.org The sequences were screened to cover the same genetic space and filtered to remove columns without alignment data, upon which the sequences were pre-clustered to remove bad sequences with 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) against a RDP reference file version 10 included in mothur, and any contaminants (sequences classified as mitochondria, chloroplasts, archaea, eukaryote or unknown) were removed. To obtain operational taxonomic units (OTUs) at the 0.03 level, we first constructed a distance matrix (cut-off 0.15), clustered the resulting sequences into OTUs and then classified them to obtain their consensus taxonomy. Sequence data (raw sff files) have been uploaded to the GenBank SRA database under accession number SRP059050

2.4. Statistical analysis

In order to remove the effect of sample size on community composition, samples were rarefied to 1178 sequences. We infer an approximately-maximum-likelihood phylogenetic tree with FastTree 2.1 (Price et al., 2010). Taxonomic alpha diversity was calculated as the observed number of OTUs (sobs), estimated diversity (Shannon index) and richness (Chao1 index). Phylogenetic diversity was calculated as Faith's phylogenetic diversity. The effect of manure on both the taxonomic and phylogenetic alpha diversity of bacterial communities from casts was assessed by one-way ANOVA tests over linear models where manure type (pig, horse and cow) was fixed as factor. For each variable we checked normality of residuals and homogeneity of variance across groups. Post-hoc comparisons were performed with Tukey test and the Benjamini–Hochberg FDR multiple test correction method was applied (library multcomp; Hothorn et al., 2008).

Taxonomic beta diversity was estimated as differences in bacterial taxonomic community composition at the OTU level between samples of casts. This was done by principal coordinate analysis (PCoA) with Bray–Curtis (considering abundance of OTUs) and Jaccard (not considering the abundance of OTUs) distance matrixes. Phylogenetic beta-diversity was also calculated by PCoA with weighted (considering abundance of OTUs) and unweighted unifrac distances (Lozupone et al., 2007), which were obtained as averages after sampling the phylogenetic tree 1000 times. All PCoAs were done with function ordinate from library phyloseq