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Diet-related composition of the gut microbiota of *Lumbricus rubellus* as revealed by a molecular fingerprinting technique and cloning

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ABSTRACT

Interactions between earthworms and microorganisms are essential for the functioning of soil ecosystems as they affect organic matter degradation and nutrient cycling. This is also true for the alpine region, where socio-economic changes lead to the increasing abandonment of pastures, which in turn, causes a considerable shift in the diet of saprotrophic invertebrates and thus impacts food web and decomposition processes. To enhance our understanding of how this diet shift influences earthworms and associated microorganisms, we studied the gut content and cast microbiota of *Lumbricus rubellus* (Lumbricidae, Oligochaeta), a key macrodecomposer on alpine pastureland in the Central Alps. A feeding experiment with *L. rubellus* and three different food sources that represent the vegetation shift from an alpine pasture to an abandoned site was set up. Earthworms were collected in the field, transferred to a climate chamber and fed with cow manure, dwarf shrub or grass litter for six weeks. PCR-DGGE (Polymerase chain reaction-denaturing gradient gel electrophoresis) analysis of the DNA extracted from the substrates, the earthworms' gut contents and casts revealed that the gut and cast microbiota was strongly influenced by the food source ingested. Cloning of bacterial 16S rRNA gene fragments demonstrated that the intestinal content was dominated by Proteobacteria, especially from the Gamma-subclass, followed by members of the phyla Bacteroidetes, Actinobacteria and Firmicutes. In contrast, Actinobacteria were detected abundantly in all samples types when a cultivation approach was used. In conclusion, the gut microbiota of *L. rubellus* was shown to be substantially affected by the food source ingested, suggesting that this essential macrodecomposer is exposed to the diet shift resulting from a land-use change in the alpine area.

1. Introduction

Earthworms are essential members of the soil macrofauna, altering the biological activity and physical structure of soils and stimulating organic matter degradation (Brown et al., 2000; Görres et al., 2001; Brown and Doube, 2004). Decomposition processes are either directly affected by the earthworms' ability to process considerable amounts of plant litter or indirectly by the enhancement of microbial activity (Hättenschwiler et al., 2005). These important functions of earthworms can also be observed on alpine pastureland, where the epigeic/hemiedaphic earthworm *Lumbricus rubellus* acts as key macrodecomposer (Seeber et al., 2005).

Over the last years alpine pastures have increasingly been abandoned due to socio-economic reasons (MacDonald et al., 2000). As a consequence, litter composition, quality and quantity is altered due to the rise of perennial grasses and dwarf shrubs and organic material accumulates on the soil surface. In contrast to managed pastures, on which cow dung as pre-decomposed organic material displays a highly attractive food source for earthworms (Doube et al., 1997; Marhan and Scheu, 2005), dwarf shrubs (such as *Vaccinium gaultheroides*) produce recalcitrant litter (Chapman et al., 2006). Although dwarf shrub litter was considered to be of low nutritional quality, *L. rubellus* was found to utilise it as food source on abandoned sites (Seeber et al., 2006, 2008). Microorganisms are believed to play an essential role in this adaptation process, especially the ones passing through the gut of *L. rubellus* and thereby facilitating digestion.

Previous studies dealing with the effect of gut passage on microorganisms were mostly based on classical cultivation methods (Krištůfek et al., 1992; Pedersen and Hendriksen, 1993; Karsten and Drake, 1995, 1997), thereby excluding the larger part of non-cultivable microorganisms from the analysis. In recent years,

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molecular tools have been implemented to characterise uncultured members of the gut microbiota (Cai et al., 2002; Furlong et al., 2002; Schönholzer et al., 2002; Singleton et al., 2003; Egert et al., 2004). Denaturing gradient gel electrophoresis (DGGE; Muyzer et al., 1993) has frequently been applied to study the microbial composition of the intestinal tract of animals, among them diplopods (Knapp et al., 2009), fruit flies (Behar et al., 2008), locusts (Dillon et al., 2008), termites (Tanaka et al., 2006) and wasps (Reeson et al., 2003). To identify members of the community, bands can be excised from the DGGE gel and sequenced; however, this way only the most dominant microorganisms can be detected and the obtained sequence information is limited (Handschur et al., 2005). In contrast, a cloning approach is more time-consuming, but does allow for the detection and phylogenetic identification of the entire 16S rRNA gene sequence of the bacteria involved (Schabereiter-Gurtner et al., 2001; Liang et al., 2008). This is why the combined application of a fingerprinting method and molecular cloning is valuable for providing a deeper insight into the gut microbial community.

The aim of this study was to investigate if and to what extent the gut microbiota of *L. rubellus* is impacted by a radical diet shift. Three naturally occurring and abundant food sources on managed and abandoned alpine pastureland differing in their palatability were chosen for the feeding experiment, simulating the ecosystem changes that occur when alpine pastures become fallow. The microbiota of the earthworms' intestinal contents as well as the casts and the organic substrates were analysed using a molecular fingerprinting method. To get more detailed insights into the gut microbial community, bacterial clone libraries were constructed and compared to a classical cultivation approach. The hypothesis underlying the present study was that the structural community composition of the microorganisms associated with the gut contents and casts of *L. rubellus* reflects the diet shift in a changing environment.

2. Materials and methods

2.1. Feeding experiment

Earthworms, soil and litter samples were collected in autumn 2006 on a managed and an abandoned alpine pasture at the Kaserstattalm (Stubai Valley, Tyrol, Austria, 47°07,529′N 11°17,391′E) and were immediately used for the feeding experiment. Three plastic boxes (495 × 380 × 250 mm; 30 l total volume) were filled with sieved (4 mm) alpine soil (C-content: 3.45%, N-content: 0.24%). As food sources either air-dried dwarf shrub litter (*V. gaultheroides*), grass litter (*Luzula* sp.) or cow dung were put on top. Afterwards ten *L. rubellus* specimens were transferred into each of the plastic boxes and incubated in a climate chamber at 12 °C for six weeks, which corresponds to the average soil temperature on the research site during the vegetation period. During incubation, fresh substrate was added regularly to provide the earthworms with sufficient food, but prevent the substrate from moulding. For obtaining cast samples, earthworms were taken from the boxes and placed on sterile filter paper moistened with distilled water. Casts were picked immediately after casting and transferred to sterile 1.5 ml microcentrifugation tubes. Afterwards, samples of the remaining substrate were collected at six randomly selected spots per box with a sterile spatula and filled into sterile 1.5 ml microcentrifugation tubes. Earthworms were sedated by fumigating them with CO_2 in a closed box, surface sterilised with ethanol (70%) and dissected from the posterior end of the intestine under sterile conditions. The gut content was extracted by scraping the gut walls off with a sterile spatula and transferred into sterile 1.5 ml microcentrifugation tubes. Each gut sample was separately used for DNA extraction in order to obtain independent replicates. The gut content samples were designated ICD (intestinal contents of earthworms fed with cow dung), IDS (intestinal contents of earthworms fed with dwarf shrub litter) and IGL (intestinal contents of earthworms fed with grass litter); cast samples were named CCD (casts of earthworms fed with cow dung), CDS (casts of earthworms fed with dwarf shrub litter) and CGL (casts of earthworms fed with grass litter); substrates were labelled with CD (cow dung), DS (dwarf shrub litter) or GL (grass litter).

2.2. DNA extraction

DNA extraction from the *L. rubellus* casts (10 samples per treatment) and gut contents (10 samples per treatment) as well as from the substrates (6 samples per treatment) was performed using the PowerSoil DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, USA) according to the manufacturer's protocol, with the following modifications to improve DNA yield but reduce shearing of large fragments: after an abbreviated bead beating step (5 min), samples were frozen at −80 °C for 1 h and thawed in a water bath at 37 °C for 30 min. This freezing-thawing step was repeated once, before continuing with the regular protocol. DNA yield and quality was assessed by 1.0% (w/v) agarose gel electrophoresis, followed by DNA concentration measurements using PicoGreen dsDNA quantitation reagent (Invitrogen, Carlsbad, USA) (Juen and Traugott, 2005) and an anthos Zenyth 3100 multimode reader (anthos Mikrosysteme GmbH, D), as described by the manufacturer.

2.3. PCR-DGGE analysis

Extracted DNA was amplified in a PCR thermocycler (PCR Express, ThermoHybaid) with different primer sets. Each PCR mixture contained 0.5 ng of extracted DNA, 0.2 μM of each primer, 0.625 U Bio Therm™ DNA Polymerase (Gene Craft), 1× DNA polymerase buffer, 0.1 μg μl^{-1} Bovine serum albumin (BSA), 4% (v/v) Dimethylsulfoxide (DMSO), 0.2 mM dNTP-Mix and 2.5 mM $MgCl_2$ in a final volume of 25 μl.

To amplify total bacterial communities the universal 16S rRNA primer set 63f (Marchesi et al., 1998) and 1378r (Heuer et al., 1997) was used as first step. The PCR included an initial 3 min denaturation at 94 °C and was followed by 30 thermal cycles of 1 min at 94 °C, 1 min at 62 °C and 2 min at 72 °C. Amplification was completed with a final extension step at 72 °C for 10 min. PCR products of the first amplification were used as template for a second PCR performed using primer pair 338fGC and 518r (Muyzer et al., 1993). The PCR program for these primers differed by an annealing step of 30 s at 56 °C.

After amplification PCR products were checked by electrophoresis in 1.5% (w/v) agarose gels and ethidium bromide staining (10 mg ml^{-1}), as well as quantified using PicoGreen dsDNA quantitation reagent (Invitrogen, Carlsbad, USA) (as described in Chapter 2.2).

Denaturing gradient gel electrophoresis (DGGE) was performed with the Ingeny PhorU2 system (Ingeny International BV, The Netherlands). Sixty nanogram of PCR product were loaded on to 7% (w/v) polyacrylamide gels with a denaturing gradient of 40–70% (100% denaturant according to 7 M urea plus 40% formamide in 1× TAE-buffer) and were run for 16 h at 60 V and at a constant temperature of 60 °C in 1× TAE-buffer (pH 7.4). After electrophoresis, the gels were stained with silver nitrate (Sanguinetti et al., 1994) using an automated gel stainer (Amersham Pharmacia Biotech, Germany), photographed and air dried for storage. After screening all gut content samples on one gel, four samples per treatment were randomly chosen and run on a DGGE gel together with four cast samples and three substrate samples per treatment