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Microbial diversity associated to the intestinal tract of soil invertebrates

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ABSTRACT

Interactions between saprophagous invertebrates and microbes are essential for the maintenance and functioning of soil ecosystems, as they directly affect the degradation of organic matter and the nutrient cycle. The intestinal tract of invertebrates is inhabited by a diversity of microbes, and it is closely associated with the food ingested. The aim of this work was to evaluate the profile of prokaryotes associated with the intestinal tract of three invertebrate species. The species of invertebrates *Trigoniulus corallinus* was collected and incubated in the experiment, after 5 days of incubation we observed the uninduced colonization of two invertebrate species *Cubaris murina* and *Pycnoscelus surinamensis*. Therefore, the three species were evaluated in the same way, after 60 days of incubation. The diet supplied comprised different vegetal residues, with distinct carbon/nitrogen compositions. Six treatments were evaluated. After 60 days, five individuals of each species were randomly selected, by removing the posterior third of the intestinal tract. These specimens were next subjected to DNA extraction. The PCR/DGGE analysis was carried out using the 16S rDNA, for the domain Bacteria and the phylum Actinobacteria. DGGE bands were cloned and sequenced using the Bacterial domain. In multivariate analyzes, individuals of the same species after 60 days of incubation, were strongly grouped. These results may be in accordance with the environmental criteria of the host itself, stage of development, phylogeny and diet. Thus, the investigation of the intestinal microbiota, provides relationships between invertebrates and their intestinal bacterial communities. In view of this information, we used the technique of sequencing cloned DGGE bands to quantify the diversity of microorganisms present in the intestinal tract of the studied invertebrates. The phylum Firmicutes, Bacteroidetes, and Proteobacteria were identified by sequencing the cloned bands; Proteobacteria presented the highest number of genera, comprising *Enterobacter*, *Buttiauxella*, *Serratia*, *Kluyvera*, and *Pantoea*.

1. Introduction

Soil microbial communities differ significantly (Robe et al., 2003), and present the highest levels of prokaryotic diversity among known habitats (Roesch et al., 2007). Thus, it is important to study ecological aspects in order to investigate the associations of microbes and invertebrates inhabiting the soil.

In general, diversity and evolutionary success of the invertebrates partially relies on innumerable associations with beneficial microbes that are known to optimize nutrient-poor diets, to assist in digesting recalcitrant food components, protect against predators, parasites and pathogens, and contribute to inter- and intraspecific communication (Engel and Moran, 2013). Processes shaping this association are central questions in research, although still poorly understood. The microbiota might co-evolve with its hosts and establish a close relationship (Koch et al., 2013). In these associations, the host immune system can actively shape the microbiota, while microbiota components might adapt, in turn, to different hosts and environments, and provide potentially important functions to the host itself (Ochman et al., 2010; Bevins and Salzman, 2011; Frese et al., 2011; Brucker and Bordenstein, 2011).

Investigating symbiotic relationships between microbes and invertebrates is one of the main fields of soil microbial ecology. Symbiosis involves the coexistence of two or more species, with the highest degree of association, both outside and inside tissues and organs (Byzov et al.,

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2009).

The digestive system of soil invertebrates contains evolutionarily diverse microbes. This implies the occurrence of several associations, one of which is responsible for the maintenance of the soil ecosystem, with processes of organic matter decomposition and nutrients cycle. A well-known example is the symbiosis of the digestive tract of termites and their function (Li et al., 2006). Besides termites, studies of symbiosis of other species of invertebrates with microbes have also been carried out in oligochaetes, diplopods, isopods, and others (König and Varma, 2006).

Microbial colonization acts as an indicator of high-quality food, thereby stimulating consumption (Zimmer et al., 2003). This may be due to microbial activity enhancing palatability and nutritive quality of the leaf litter (e.g.,by decreasing the C:N ratio and the content of phenolic compounds) prior to ingestion by isopods (Bouchon et al.,2016).

Changes in the use of the land and vegetable waste supply alter invertebrates' diet, which will consequently change the population of microbes that can be found in the intestinal tract as well as the rates of decomposition process. However, the nutritional interactions of the intestinal microbiota can increase invertebrates' survival, against optimal diets. This improves digestive efficiency, and provides digestive enzymes and vitamins (Dillon and Dillon, 2004).

The plant tissues present in their composition cellulose representing more than 50% of the foliage and > 90% of the woody tissues. The physical disruption of plant material by chewing insects increases the availability of cellulose to enzymes (Douglas, 2009). Distribution of cellulase genes among insects has yet to be explored in detail either from a phylogenetic perspective or in relation to insect feeding habits (Douglas, 2009). Plant-parasitic nematodes, cockroaches and termites were among the first to be proven to carry cellulase genes, recently these genes have also been unambiguously demonstrated in other taxa, such as other insects, Gastropoda, Crustacea and Annelida (Cragg et al., 2015).

Given that the diet is considered one of the main factors determining the populations of gut microbes in invertebrates (Ley et al., 2008; Staubach et al., 2013), the aim of this study was to investigate the structure of bacterial community in three species of saprophagous invertebrates, important in the fragmentation of plant residues and decomposition of organic matter. These are: the millipede *Trigoniulus corallinus*, the terrestrial isopod *Cubaris murina* (Brandt, 1833), and the cockroach *Pycnoscelus surinamensis* (Linnaeus, 1758). It is important to report that the initial goal of this work was to investigate only the structure of the bacterial community in one invertebrate species, the millipede *T. corallinus*, which was collected in compost piles, incubated, and fed with legumes, grasses, and recalcitrant materials. However, two weeks after the start of the experiment, the spontaneous colonization of *C. murina* (Brandt, 1833) and the cockroach *P. surinamensis* was observed (Linnaeus, 1758).

In order to relate the bacterial structure to the diets provided, a multivariate analysis was performed using the main components (PCA), while cloning analysis was subsequently carried out on bands extracted from the DGGE gel.

2. Materials and methods

2.1. Invertebrate collection and intestinal tract extraction

Invertebrates were obtained from an Embrapa Agrobiology field experiment at the Integrated Agroecological Production System (IAPS), located in the municipality of Seropédica, RJ, located between the parallels 22° 49′ and 22° 45′ S and the meridians 43° 23′ and 43° 42′ W, in average altitude of 33 m, in the Baixada Fluminense. The climate of the region, according to the classification of Köopen, is of type Aw (Tropical Climate with dry season). The soil of the experimental area was classified as Red-Yellow Argissolo (EMBRAPA, 2006).

These experiments were repeated twice, with the aim to compare the data. Both experiments were used, with different organic residues, lignocellulosic compositions, and distinct carbon/ nitrogen (C/N) ratios. The residues that were added were based on legumes: Flemingia (*Flemingia macrophylla*) (*Willd.*), Gliricidia (*Gliricidia sepium*) (*Jacq*), bahiagrass (*Paspalum notatum*) (*Flügge*), and recalcitrant materials such as cardboard, corncob, and coconut fiber. The latter were subsequently supplied to and consumed by diplopods of the species *Trigoniulus corallinus* (Gervais, 1847; Diplopod: Spirobolida). In this experiment, 500 mL of *T. corallines*, which account for approximately 900 individuals, were intentionally added as composting agents of these residues, for a period of 60 days, under controlled humidity and ambient temperature conditions. The other two species of invertebrates that were investigated in this study, *Pycnoscelus surinamensis* (Linnaeus, 1758) (Blattodea: Blaberidae) and *Cubaris murina* (Brandt, 1833) (Crustacea: Isopoda), spontaneously colonized the experiment. As shown in Table 1, the experiment consisted of six treatments with four repetitions, carried out in blocks and randomly distributed.

Table 1
Treatments of vegetable residues supplied to the diplopod of the species *Trigoniulus corallinus* (Gervais, 1847).

Treatments	Legumes	Grass	Recalcitrant materials	*C/N ratios
T1	*Flemingia* 30%	*Paspalum* 40%	cardboard 30%	134,85
T2	*Flemingia* 30%	*Paspalum* 40%	corncob 30%	46,53
T3	*Flemingia* 30%	*Paspalum* 40%	coconut fiber 30%	47,63
T4	*Gliricidia* 30%	*Paspalum* 40%	cardboard 30%	127,67
T5	*Gliricidia* 30%	*Paspalum* 40%	corncob 30%	39,36
T6	*Gliricidia* 30%	*Paspalum* 40%	coconut fiber 30%	40,45

* C/N: Carbon/Nitrogen.

Five individuals of each species were randomly selected per treatment, according to the methodology of Tokuda and Watanabe (2007). These were anesthetized in ether for 10 min, disinfested superficially with 70% alcohol, and dissected with the aid of a magnifying glass. The entire digestive system was harvested; the hindgut was sectioned, and immersed in 1.0 mL of Ringer's solution: 47 mM NaCl, 183 mM KCl, and 10 mM Tris-HCl, at pH 6.8 (Cazemier et al., 1997).

2.2. Extraction of DNA from the bacterial community associated with the intestinal tract of saprophagous invertebrates

After harvesting the hindgut, a sample of about 1 g was removed to extract DNA from adhered microbes, and placed in 1 mL of Ringer's solution. The samples were vortexed for 30 s at full speed, left for 15 min in a refrigerator, and sonicated for 45 s. The microtubes were centrifuged for 15 min at 9300*g*. The supernatant was discarded, and the pellet was subjected to DNA extraction, performed using the commercial kit "Ultra Clean Soil DNA Isolation Kit" (MOBIO), according to the manufacturer's instructions. The extraction efficiency of purified genomic DNA was confirmed by electrophoresis, running the samples in a 1% agarose gel.

2.3. PCR amplification

The analyses of the structures of the bacterial community found in the intestinal tract of the studied saprophagous organisms were carried out by PCR-DGGE. For the domain Bacteria, total DNA template was used to amplify the 16S rDNA variable region V6 - V8 using 968GC and 1401R primers (Heuer et al., 1997) (Table 2). The PCR reaction was performed in a final volume of 30 µL, which contained 3 µL of DNA template, 1.25 µM of $MgCl_2$, 0.25 µM of dNTP, 2.5 U of *Taq* DNA polymerase enzyme, Kit PCR - Master Mix 2X (Promega), and 0.2 µM of each primer. The reaction was conducted in a thermocycler under the following conditions: initial denaturation at 93 °C for 5 min, 35 cycles of denaturation at 93 °C for 1 min, annealing at 62 °C for 1 min, extension

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