IDENTIFICATION OF THE INTESTINAL MICROBIAL COMMUNITY OF GLOSSOSCOLECIDAE EARTHWORMS (ANNELIDA: OLIGOCHAETA)

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ABSTRACT: Earthworms play an important role in soil ecology. Their actions are favored by the activity of the microbial communities that inhabit the alimentary canal of these organisms. The main aim of this work was the identification of the microbial flora of the alimentary canal of native earthworms species of Glossoscolecidae (*Enantiodrilus borelli* and *Pontoscolex corethrurus)* of the Neotropical region. The animals were collected in Chicligasta, Tucumán, Argentina. The alimentary canal was removed by dissection and later macerated in physiologic solution. The stumps were isolated and identified by standard microbiological procedures on the bases of their morphological and biochemical characteristics. Twelve samples were isolated and ten identified, among them *Marinococcus* sp., *Caryophanon* sp., *Planococcus* sp., two stumps of *Oscillospira* sp., *Bacillus* sp., two stumps of *Pseudomonas* sp., *Acidiphilium* sp., *Azomonas* sp. These earthworms were found to have a wide and diverse microbial flora in the alimentary canal, with both, endogenous communities and some that entered the worm by ingestion.

KEY WORDS: Intestinal microorganisms, diversity, mutualism, Glossoscolecidae, soil.

The earthworms play an important role in the terrestrial ecosystem, contributing to the fertility of the soil, modifying the physical, chemical and biological properties (Edwards & Lofty, 1972; Syers & Springett, 1984; Krishnamoorthy, 1985; Hoogertkamp et al., 1983; Lavelle, 1983; Lavelle & Martin, 1992; Egert et al., 2004). This is shown by having found high quantities of organic carbon, nitrogen, inorganic phosphorus, potassium and magnesium among others, in the excrete or worms casts (Burk et al., 1999; Schrader & Zhang, 1997; Brussaard et al., 1996; Kolmans & Vásquez, 1996).

Therefore, it was verified the bacteria existence in the alimentary canal of *Lumbricus terrestris* and that some of them were not in the surrounding soil (Bassalik, 1913), it is also known that the microflora of the surrounding soil forms part of the diet of the intestinal worm. Some of them are not affected by the digestive enzimes. It was suggested that probably some of these enzimes are produced by the microorganisms in the alimentary canal of the eathworms.These enzimes provide them a better ability to degrade the complex substances foud in the organic substance of the soil (Lee, 1985; Edwards & Fletcher, 1988; Fukatsu & Nikoh, 1998; Santiago, 1995; Alonzo et al., 1999; Vinotha et al., 2000). That is why it is suggested , in works of Hubers et al. (1993) and Hubers (1993), the existence of a mutualist relation between a earthworm species *Onychochaeta borincana* and the microorganisms of the soil, postulating that this fact would allow the annelid to adapt to different habitats.

The diversity of bacterial community within the digestive tract of earthworms depends on climate, soil type and organic mather (Brito-Vega & Espinosa-Victoria, 2009).

Other investigations show the determination of the communities of microorganisms that are endogenous of every species of earthworms (Toyota & Kimura, 2000) to distinguish them from those that could be inoculated or consumed with the soil where they live.

Earthworms have been studied during the last years due to their importance in areas such as biotechnology (vermicomposting process using earhworms in breakdown sludge (Naddafi et al., 2006; Alidadi et al., 2007); search of potencial species of earthworms for vermicomposting process (Verma & Shweta, 2011)), ecotoxicology (relationship of earthworms and plants to Pb phytoextraction (Huynh et al., 2011), ecology (predominance of native species in area possibly due to low of disturbace in biophere reserve (Mohan et al., 2011) and identificacion of species gratheres from the geographic regions (Yousefi et al., 2009)), taxonomy and morphology (earthworm species identification by morphological characters (Omrani et al., 2005; Ansari & Saywack, 2011)), soil physics and soil fertility (Amador & Gorres, 2007; Aira et al., 2002; Fraser et al., 2003; Homa et al., 2003; Spurgeon et al., 2003). The earthworms are good bio-indicators for forest site quality and are thus useful when planning forest production improvement (Kooch et al., 2008).

Earthworms have many bacterial diversity within their digestive tracts and it is very little explored mainly because of the non-cultivatable character of a large quantity of microorganisms which mainly come from soil. Soil is an appropriate environment for the development of eukaryotic (algae, fungi, protozoa) as well as prokaryotic (bacteria and archaeas) microorganisms. Virus and bacteriophage are also present (Nogales, 2005). All these organisms establish relationships among themselves in highly varied and complex ways that contribute to soil characteristics because of their role in the rnodification of solid, liquid and gaseous stages.

The bacterial community inside the digestive tract of earthworms pertains to at least four physiological groups: plant growth promoters, free-living nitrogen fixers, biocides and phosphate solubizers.

Undoubtedly it exists very little information of taxonomic level of bacteria found in the digestive tract of earthworms. Especially in those animals that have a wide geographical distribution and can accommodate a wide range of soil types as well as different land-intensive crops altered by man-made.

That is why the aim of this study was to determine in a broad sense the community and soil microflora where the specimens of two species of earthworms of the family Glossoscolecidae (*Enantiodrilus borelli* and *Pontoscolex corethrurus)* are found. In this way we try to extend the knowledge on common species in our region and which are characterized by ability to resist modified environments such as cultivated soils or damaged by aggressive agricultural practices.

MATERIALS AND METHODS

The worms were obtained by means of collections made in April 2007 and May 2008, in a sugar cane farm, located in the Chicligasta Department, at the height of 745 km of national route nº 38 (S 27 ° 29 ' 26''-W 65 ° 37 '18'') to 370 m s.n.m., in the Tucumán province, Argentina.

On site, soil samples were taken and animals were removed from two species of earthworms of the family Glossoscolecidae: *Pontoscolex corethrurus* (Muller, 1857), identified by descriptions of Moreno (2004) and Gates (1973) and Enantiodrilus borelli (Cognetti, 1902) identified as works of Moreno et al. (2005).

This family of Oligochaeta is endemic to the Neotropical region and both species listed above are considered cosmopolitan, associated with human activities and supporting changes in soil conditions caused by disorders related to agricultural activities.

Earthworms were obtained manually in the collections and simultaneously samples of soil were obtained at a depth between 5 and 15 cm, discarding the litter layer. All samples were transported to the laboratory in black plastic bags, protected from light, kept in a moist environment at an ambient temperature between 15-20 ºC.The separation and identification of the worms took place in the laboratory.Then the dissection was performed on 5 specimens of each species in order to remove the portion of intestine to detect micro-organisms. The worms were numbed by cold in the refrigerator at 7ºC.Then a cut of approximately 15 segments in length starting from the clitellum was performed in the ventral area. It allowed to expose the digestive tract to make the cuts dissecting a section of 10 segments in length starting from the clitellum toward the rear of the body. Once removed the sections of the digestive tract plus its contents, they were macerated in saline solution and then proceeded to carry out successive dilutions.

Colonies were isolated in Petri dishes with nutrient agar solid overall average, according to the technique eventually led striatum and incubated under aerobic conditions in an oven at 30 ºC. Representative colonies emerging from the plates were grouped according to their cultural characteristics, purified by repeated sub-cultur- ing and maintained on appropriate agar slants as stock cultures.

These strains were marked and then were exposed to different tests for identification: Gram reaction (Claus, 1992), morphology, motility, cysts, zoogloeas, endospores produced, color of colonies, color change of the media, catalase and oxidase reactions, growth with different concentrations of NaCl, variable temperature and pH, fix N_2, poly β-hidroxybutirato accumulated, oxidize etanol to acetic acid, citrate, Indole and Sulfate utilization and sugar fermentation (Harrigan & McCance, 1976; Seeley& Van Demark, 1972).

The isolates were then identified with reference to the Manual of Bacteriological Determination of Bergey (Holt et al., 1994).

RESULTS

The results of the identifications made with the techniques used (Table 1) are described below:

For *Pontoscolex corethrurus* of sowing crops all 12 samples were isolated from the intestinal contents, we identified 10 of these samples and the remaining two samples were as dubious. Identifications were found in 4 strains of *Micrococcus* spp; 1 strain of *Marinococcus* sp, 1 strain *Enterococcus* sp and 4 of *Pseudomonas* spp.

For of samples of the *Enantiodrilus borelli*, 12 samples were also isolated from the intestinal contents, identifying only 10, leaving 2 samples as doubtful. In this case they were: 1 strain of *Marinococcus* sp., 1 of *Caryophanon* sp., 1 of *Planococcus* sp., 2 strains of *Oscillospira* spp., 1 of *Bacillus* sp., 2 strains of *Pseudomonas* spp., 1 of *Azomonas* sp. and 1 strain of *Acidiphilium* sp.

In the analysis of soil bacterial flora were isolated from 11 samples, which are able to identify the following bacterial genera: 1 strain of *Micrococcus* sp, *Marinocccus* sp., *Planococcus* sp. and *Caryophanon* sp., 3 strains of *Sporohalobacter* spp. and 4 strains of *Pseudomonas* spp.

In comparing the results obtained from the intestinal contents of the two earthworm species studied and the identifications of the soil samples, it was possible to differentiate the bacterial strain that is found exclusively in the gut (endogenous) of these animals. In *Pontoscolex corethrurus*, the strain of *Enterococcus* sp. is found exclusively in the intestine and is considered endogenous, other strains were found in both the ground and in the digestive tract. As in the case of *Enantiodrilus borelli* strains were only found in the intestine were *Azomonas* sp., *Acidiphilium* sp., *Oscillospira* spp. and *Bacillus* sp. and are considered endogenous, the other strains were found both in the ground, as in the intestine.

DISCUSSION

The results of our study show a high bacterial diversity compared with the results of investigations of other authors, such as those made by Santiago (1995) who studied the intestinal bacterial flora of *Onychochaeta borincana* (Glossoscolecidae), in this case has identified only the genus *Bacillus* with 7 different species. It is important and valid the comparison of our results with those obtained in the work of Santiago (*op cit.*) since both techniques have been used considered basic microbiological culture. The genus *Bacillus* has been found by us in the gut contents of *Enantiodrilus borelli*, along with other strains, but it is curious that this strain was not identified in the soil where crops are worms. On the other hand, this time with the use of different methods and laboratory techniques (basic microbiological techniques, DNA testing, cleaning of the bowel and scanning electron microscopy) Valle Molinares (2006) identified in the intestinal microflora of *O. borincana* the same genus but a different species, *Bacillus cereus*. Although, Kumar & Shweta (2011) studied the microbial load in *Eisenia foetida* and their association with vermistabilization. Which was identified *Pseudomonas* sp., *Stretosporangium* sp., *Salmonella* sp., *Shigella* sp., *Flexibacter* sp. and *Escherichia* sp. in gut and casts.

When the comparison was made of the results obtained in the identification of the strains found in soil with the earthworm gut contents, we confirmed that the microorganisms found in the digestive tract of earthworms are some of them different. Thus, this difference could be attributed to the presence of an endogenous microflora, thus identify strains of *Enterococcus* sp., *Azomonas* sp., *Acidiphilium* sp., *Oscillospira* sp. and *Bacillus* sp. and consider them as endogenous bacteria of the two earthworm species studied. Our results are similar to those of Mendez et al. (2003) which support the concept that there are gut bacteria considered as endogenous and which are not found in the soil where they live, also show that with a pre-wash these bacteria from the digestive tract remain intimately associated to the inner wall of the intestine.

Idowu et al. (2006) identified the intestinal bacterial flora of *Libyodrillus violaceous*, an earthworm native of the Nigeria through basic microbiological testing, the same as those used in our work and found: *Staphylococcus*, *Bacillus* spp., *Pseudomonas aeruginosa*, *Streptococcus mutans*, *Clostridium*, *Spirocheata* spp., *Azotobacter* spp., *Micrococcus lyla*, *Acinetobacter* spp. and *Halobacterium* spp. When we compare these results with ours, we only find agreement in three genera, we identified as *Bacillus* sp., *Pseudononas* sp. and *Micrococcus* sp., of which only one, *Bacillus* sp., we consider as endogenous in the earthworms with which we work. All this leads us to the interpretation that there is no bacterial flora characteristic of each species and therefore the current research can not be said that the endogenous microflora is specific to each species of earthworms. However, at present there are few and far between studies, this implies that, in later stages of the knowledge of intestinal bacterial flora earthworms this situation could be reviewed, with results that enable comparisons using similar methodologies.

Each author used different methodologies for the identification of bacterial strains, we believe that in the context of our research, the methodology used in this study is appropiate for the identification of bacterial strains to genus level. Future studies that merit further identifications to species level should turn to techniques with greater specificity and resolution, such as molecular techniques and scanning electron or tranmission microscopy, among others.

The bacteria were identified in this work are known to perform different functions in each place where they are, why could be explained by extrapolation, the role fulfilled in the different environments where found (soil, intestine). *Pseudomonas* sp., *Micrococcus* sp. and *Bacillus* sp. among others, promote germination, growth, vegetative development, nitrogen fixation and increase the absorption of nutrients, including nitrogen, phosphorus and potassium, are also capable of producing the phytohormone, in addition to serving as a biocontrol of phytopathogenic fungi . These strains are considered of great importance for their potential as biofertilizers for agriculturally important plants (Diaz Vargas et al., 2001; Beringer & Johnston, 1984; Ferrera-Cerrato, 1995; Rodriguez, 1995; Chanway et al., 1989; Knudson, 1922; Wilkinson et al., 1989, 1994; Peter et al., 1987; Alexander, 1981; Asea et al., 1988, Salih et al., 1989; Tsavkelova et al., 2004, 2005; Fuentes et al., 2003; Jimenez-Salgado et al., 1997). In particular, it was mentioned that the amylolytic function in soil and water bodies in charge of the genus *Bacillus* sp. as well as the proteolytic and lipolytic role in charge of the genus *Pseudomonas* sp., which is often found in environments rich in organic matter (Cardenas, 1995).

That is why we proposed the use of oligochaetes for studies focusing on the evaluation of abnormalities and identification of different types of soil (Rombke & Jänsch, 2004).

The bacteria of the genus *Sporohalobacter* sp. can be used as bioremediation of soils with a variety of contamimantes (Oren et al., 1991). This strain was found in soil, reflecting its beneficial role by reducing the polluting effects of chemicals used in the field and land suitable for promoting the development of soil fauna.

Other strains identified in this work, some of which are not as common, meet various agronomic features. *Enterococcus* sp. in combination with *Lactobacillus* sp., showing that these bacteria are capable of producing lactic acid fermentation getting high and therefore a higher nutritional value, on the other hand, lactic acid lowers the pH to function as an inhibitor of bacterial growth, mainly damaging bacteria (Seale, 2003). The bacterial genera: *Pseudomonas* sp. and *Azomonas* sp. nitrites used to make them assimilable forms in soils with human activity. Nitrite compounds are contaminants of groundwater and soil and is known to one of the main sources of nitrogen are fertilizers used in agriculture (Demakov et al., 2007). The genus *Acidiphilium* sp. inhabit extreme environments heartburn, also involved in the iron cycle and its function is redox mediator for the oxidation reactions of organic compounds (Malki et al., 2008). *Marinococcus* sp. synthesized molecules (tetrahydropyrimidines ectoine and hydroxyectoine) mainly responsible for the osmoadaptation, without interfering with the central metabolism. The accumulation of these molecules prevents dehydration of the bacteria even when exposed to high osmotic stress (Severin et al., 1992).

For all the foregoing that each of the strains of the soil microflora play an important role in soil ecology, all perform essential functions for the maintenance of conditions and its interaction with other organisms helps the development of animals and plants that inhabit and use as a substrate to the ground.

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Table 1. Bacterial isolates found in the earthworms *Pontoscolex corethrurus, Enantiodrilus borelli* and soil.

Pontoscolex corethrurus	***Enantiodrilus borelli***	**Soil**
Micrococcus sp.	X	*Micrococcus sp.*
Enterococcus sp.	X	X
Pseudomonas sp.	*Pseudomonas sp.*	*Pseudomonas sp.*
Marínococcus sp.	*Marínococcus sp.*	*Marínococcus sp.*
X	*Oscillospira sp.*	X
X	*Caryophanon sp.*	*Caryophanon sp.*
X	*Azomonas sp.*	X
X	*Planococcus sp.*	*Planococcus sp.*
X	*Acidiphilium sp.*	X
X	*Bacillus sp.*	X
X	X	*Sporohalobacter sp.*