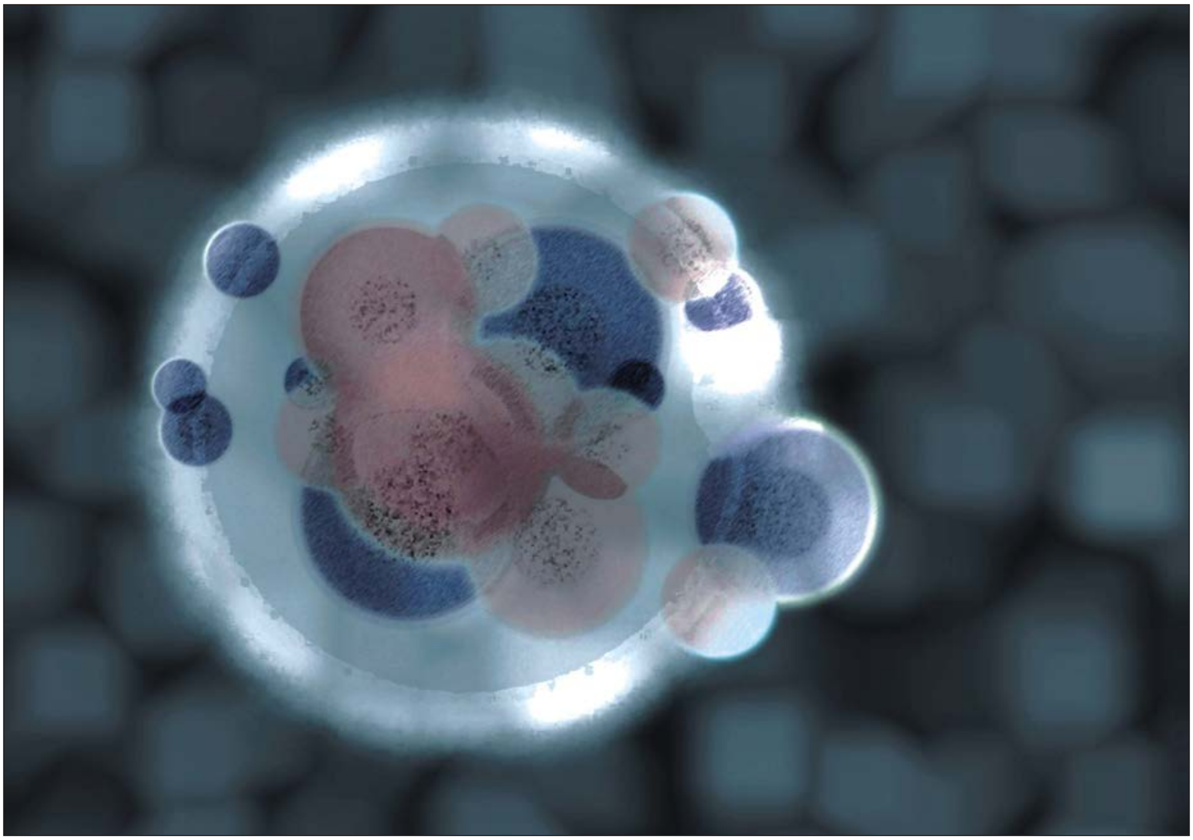

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Bacterial Diversity in the Digestive Tract of Earthworms (Oligochaeta)

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Abstract: Anecic, epigeous and endogeous earthworms stimulate or inhibit the growth of bacteria of agricultural importance inside their digestive tracts. It is possible that these bacteria establish a mutual symbiosis within the digestive tract of the earthworm. The bacterial species reported within the intestines of the earthworms belong to the genuses *Bacillus*, *Aeromonas*, *Pseudomonas*, *Flavobacterium*, *Nocardia*, *Gordonia*, *Vibrio*, *Clostridium*, *Proteus*, *Serratia*, *Mycobacterium*, *Klebsiella*, *Azotobacte* and *Enterobacter*. These bacteria inhabit the soil and develop considerably when there are easily degradable organic soil nutrients. 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 solubilizers. The diversity of bacterial communities within the digestive tracts of earthworms depends on climate, soil type and organic matter. The objective of this present study was to analyze the state of art on the bacterial diversity within the digestive tracts of earthworms.

Key words: Anecic, epigeous, endogenous, symbiosis, soil

INTRODUCTION

Anthrotropic activities such as deforestation, contamination and the advance of urban sprawls, lead the gradual disappearance of forests and other natural vegetation along with soil fauna which makes up the mega a macrofauna (Hernández-García and Granados-Sánchez, 2006; Huhta, 2002).

Earthworms are an important part of the macrofauna, visible with the naked eye (>10.000 μm) and represent 82% of the total biomass in tropical zones with a precipitation above 1000 mm (Lavelle *et al*., 2006). The diversity of these macroorganisms depends on biotic conditions and factors such as temperature, humidity, apparent density, pH and organic matter which form part of their diet (Curry and Schmidt, 2007). Currently more than 7.245 species of Oligochaetes have been classified at global level, from which 4000 earthworms species are described (Fragoso, 2001; Reynolds, 1998). In Mexico, 129 species have been identified from which 46 are native, 47 exotic and 36 others in which the majority belong to the family Megascolecidae (Fragoso, 2001).

Earthworms intervene in soil biological regulation systems, posses the capacity to remove soil particles and produce organomineral structures called biogenic structures (Rossi *et al*., 2006). They also help to maintain soil structure, water infiltration and regulate the availability of nutrients assimilated for plants, which includes nitrogen (N) in the form of ammonia (NH_4^+) and nitrates (NO_3^-) (Desjardins *et al*., 2003).

Earthworms mechanically mix mineral particles and organic matter through their digestive system which carries out disintegration, grinding and digestion of the ingested material, increasing or decreasing the activity and number of beneficial or pathogenic microorganisms (fungi, Actinomycetes and bacteria) (Winding *et al*., 1997). The participation of microorganisms within the digestive tracts of earthworms is of great importance given that a lot of these are involved in the degradation of organic matter (Byzov *et al*., 2007). For studies on bacteria within the intestines of earthworms, diverse methods and techniques have been used which have helped in identifying species of the genuses *Bacillus*, *Pseudomonas*, *Klebsiella*, *Azotobacter*, *Serratia*, *Aeromonas* and *Enterobacter* (Valle-Molinas *et al*., 2007; Byzov *et al*., 2007; Singleton *et al*., 2003). These bacteria are mainly plant growth promoters, free-living nitrogen fixers and phosphate solubilizers (Loreno-Osti *et al*., 2004; Martínez-Romero, 2001). Some researchers have indicated the existence of a possible type of mutualism between these two organisms (Brown *et al*., 2000; Barois and Lavelle, 1986). Therefore, the objective of this study was

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to carry out a revision of the state of art of the role of these earthworms and the bacterial diversity within their digestive tracts.

TAXONOMY AND ECOLOGY OF EARTHWORMS

Earthworms have been studied during the last years due to their importance in areas such as biotechnology, ecotoxicology, morphology, ecology, taxonomy, soil physics and soil fertility (Amador and Görres, 2007; Aira *et al*., 2003; Fraser *et al*., 2003; Homa *et al*., 2003; Spurgeon *et al*., 2003).

Earthworms have been classified within the phylum: Annelida, order: Oligochaeta and class: Clitella (Edwards and Lofty, 1977). The order Oligochaeta consists of 12 families: Moniligastridae, Megascoleidae, Ocnerodrilidae, Acanthodrilidae, Octochaetidae, Eudrilidae, Glossoscolecidae, Sparganophilidae, Microchactidae, Hormogastridae, Criodrilidae and Lumbricidae (Edwards and Lofty, 1977). From an agricultural point of view, the most important family is Lumbricidae and includes the genuses *Lumbricus*, *Aporrectodea*, *Allolophora*, *Dendrobaena*, *Eisenia*, *Helodrilus*, *Octalasion* and *Eophila* (Edward, 2004). The family Glossoscolecidae includes two genuses, *Periscolex* and *Pontoscolex,* which are of agricultural importance in tropical zones (García and Fragoso, 2003; Reynolds, 1998).

From an ecological point of view, earthworms have been classified in three categories: (1), epigeous, which live within the soil, interact with edaphic microbiota and modify soil profile with their underground galleries and excrements; (2) endogenous, which transform dead fallen leaves, are soil consumers and are subdivided in poli, meso and oligohumics, with reduced interaction with microbiota and (3) anecic, which lives in and consumes leaf litter (Curry and Schmidt, 2007). This classification is used in diverse studies in order to establish the ecological category of these invertebrates under different agricultural systems in the Mexican Republic (Table 1).

In Mexico 129 species of earthworms have been identified where only 10 species have been studied from a point of view based on population, soil fertility and plant growth (Fragoso, 2001). The exotic endogenous specie *Pontoscolex corethrurus* is considered geophagous and is one of the most researched species in tropical zones (García and Fragoso, 2003; Barois *et al*., 1993; Barois, 1992).

Earthworms affect processes within the soil in a direct (incorporation and redistribution of several organic and inorganic materials, aeration, moisture distribution, infiltration) or indirect manner (formation of microbial communities, transportation of propagules and inhibition of pathogens) (Byzov *et al*., 2007).

To some degree, the functions carried out by earthworms depend on the efficiency of their digestive systems which comprises of: mouth, pharynx, crop, intestines and anus (Fig. 1). These invertebrates interact with soil microorganisms, as well as biological structures

Table 1: Ecological categories of earthworm species under different agricultural systems in Mexico

Species	Category	Habitat	References
Diplocardia sp. *Apocarrectodea caliginosa* *Phoenicodrilus taste*	Endogenous, Polihumics	Cereal cultivation systems, (Aguascalientes)	Brito-Vega *et al.* (2006)
Phoenicodrilus taste	Endogenous, Polihumics	Cereal cultivation systems, (Guanajuato)	Brito *et al.* (2006)
Mayadrilus calakmulensis	Endogenous	Family Orchards (Tabasco)	Huerta *et al.* (2005)
Dichogaster saliens	Epigeous	Mango cultivation (Tabasco)	
Balanteodrilus pearsei	Endogenous	Corn and sugarcane cultivation (Tabasco)	Huerta *et al.* (2005)
Pontoscolex corethrurus	Endogenous, Poli-mesohumic	Pastorial systems with cereals (Veracruz)	García and Fragoso (2003)
Diplocardia sp.	Endogenous	Sugarcane cultivation (Tamaulipas)	Fragoso (2001)
Pontoscolex corethrurus	Endogenous	corn, sugarcane, banana, coconut cultivation and pastures (Veracruz)	
Lodrilus bonampakensis	Endogenous	Banana cultivation (Tabasco)	Fragoso (2001)
Amynthas gracilis	Epigeous	Banana cultivation (Tamaulipas)	Barois *et al.* (1993)

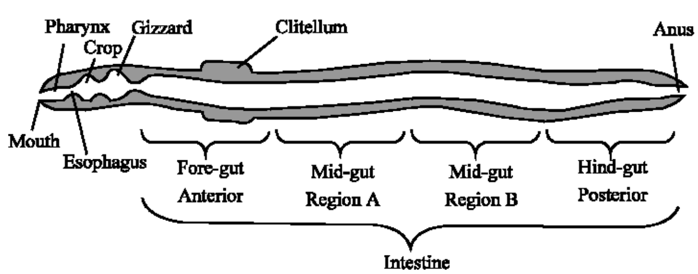

Fig. 1: Diagram of the digestive system of an earthworm (Horn *et al*., 2003)

known as turricules (feces) which they produce within the soil (Curry and Schmidt, 2007). Within their digestive systems, enzymatic activity is stimulated and may promote or inhibit the proliferation of certain fungal, actinomycetes and bacterial communities (Byzov *et al*., 2007; Barois, 1992).

DIVERSITY OF SOIL BACTERIA

Earthworms posses an immense bacterial diversity within their digestive tracts and 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 archeas) microorganisms. Virus and bacteriophage are also present (Nogales, 2005). All these organisms establish relationships among themselves in highly varied and complex ways which contribute to soil characteristics because of their role in the modification of solid, liquid and gaseous stages.

Plant beneficial prokaryotes may have a considerable potential as biocontrol agents and biofertilizers. Two large groups are distinguished: (a) nitrogen fixing microorganisms and (b) plant growth promoting bacteria. The last group of bacteria is known as PGPR (plant growth promoting rhizobacteria) or as root living bacteria which significantly stimulate plant growth (Loreno-Osti *et al*., 2004; Espinosa-Victoria *et al*., 2006).

Nitrogen fixing prokaryote microorganisms are classified into two groups: (1) obligated symbiotic, which infects the roots of legumes and (2) non-obligated symbiotics or free-living, which establishes relations with a range of gramineous plants. Within the second group, bacterial species from some genuses like *Azospirillum*, *Acetobacter*, *Azotobacter*, *Beijerinckia*, *Pseudomonas*, *Bacillus* and *Vibrio* have been reported (Bashan *et al*., 2004; Young *et al*., 2001).

Another group of soil bacteria is the Phosphate Solubilizing Bacteria (PSB) which perform an important role in supplementing di and monobasic phosphorous to plants. There are studies which have demonstrated the capacity of different species of bacteria which solubilize this insoluble phosphate and some of these bacteria belong to the genuses *Bacillus*, *Enterobacter*, *Erwinia*, *Pseudomonas*, *Rhizobium*, *Serratias*, *Agrobacterium*, *Burkholderia*, *Achromobacter*, *Micrococcus*, *Aerobacter*, *Flavobacterium* and *Erwinia* (Fernández *et al*., 2005).

Rizopheric bacteria are capable of producing physiologically active substances such as vitamins, giberelines, citochinines and Acetic Indole Acid (AIA) in significant quantities. The genuses *Azospirillum* and *Klebsiella* produce auxin (AIA), which causes morphological changes in the root and is related with the absorption of minerals mainly in corn and teocintle (Carcaño *et al*., 2006; Cattelan *et al*., 1999).

The alternative of using bacteria as a control agent against pathogens has developed certain bacterial strains with a wide spectrum of effectivity. Some species include *Bacillus subtilis*, *B. cereus*, *B. thuringiensis*, *B. cepacia*, *Pseudomonas aerufasciens*, *P. chlororhapis*, *P. corrugata*, *P. fluorescens*, *P. putida*, *Burkholderia cepacia*, *Enterobacter* sp. BF 14, *Serratia plymuthica*, *Serratia marcescens*, *Agrobacterium* sp. (De Lima-Ramos *et al*., 2004).

Soil bacteria are not randomly distributed and thus follow special aggregation patterns at different scales (from nm to km) which they superimpose. This structuring obeys the effect caused by different control factors and is completely dynamic (Ettema *et al*., 2002). Bacteria organize themselves in micro colonies comprised of few cells that may pertain to different morphotypes (Nunan *et al*., 2003).

MICROBIAL DIVERSITY WITHIN THE DIGESTIVE WITHIN THE DIGESTIVE TRACTS OF EARTHWORMS

Plant detritus in soil is the main nutritional source for earthworms although a few microorganisms like protozoans, Actinomycete, bacteria and fungi have been found to be part of the diet of these worms (Table 2) (Byzov *et al*., 2007; Hyun-Jung *et al*., 2004; Furlong *et al*., 2002). This relationship between microorganisms and Oligochaetes is not necessarily limited to a predatory process, as it has been demonstrated that the digestive enzymes produced by the earthworms do not significantly affect some microorganisms (Barois *et al*., 1993).

One of the first forefathers on microbial presence in the digestive system was Parle (1963), who reported bacterial, fungal and Actinomycetic populations in three species of earthworms; *Lumbricus terrestres*, *Allolobophora caliginosa* and *A. longa*.

Table 2: Microbial presence within the intestines of different species of earthworms

Earthworm species	No. of species of bacterias	No. of species of actinomycetes	Habitat	References
Eisenia fetida	91	-	Industrial waste	Hyun-Jung *et al*. (2004)
Lumbricus rubellus	95	-	Agricultural soil	Furlong *et al*. (2002)
Lumbricus rubellus	-	76		
Octalasion montanum	-	175	Forest soil	Kritufek *et al*. (1993)
Eisenia luceus	-	145	Forest soil	Szabó *et al*. (1976)

A study carried out by Krištůfek *et al.* (1994) found populations of bacteria, actinomycetes, fungi, sterile mycelium and plant cells in soil, however within the intestines of the earthworm *Lumbricus rubellus*, these organisms were found lysate except for a few actinomycetes, endospores and encapsulated bacteria. On the other hand, Márialigeti (1979) found that the microbial flora within the posterior segment of the intestines of *Eisenia lucens*, contained 473 organisms where 73% pertain to the genus *Vibrio*.

Contreras (1980) reported that 70% of flora in the intestines of *Eisenia lucens* was represented by only one species of Actinomycete, *Streptomyces lipmanii*, an organism rarely found in nature. On the other hand, Krištůfek *et al.* (1993) identified two species of Actinomycetes denominated *Streptomyces diastatochromogenes* and *Streptomyces noglalater*, which are characteristic of soil within the digestive tracts of two earthworm species: *Lumbricus rubellu* and *Octolasion montanum*.

Dash *et al.* (1986) did a microfungal characterization in the digestive tract of the three species of earthworms (*Ortochaetona surensis*, *Lampito maurittii* and *Drawida willsi*) found in the tropical zones of India and identified 18 species of fungi from the genuses *Aspergillus*, *Penicillium*, *Thielavia*, *Botryotrichum*, *Fusarium*, *Rhizopus*, *Curvularia*, *Chaetomium* and *Trichoderma*. Four more genuses, namely *Neocosmospora*, *Cladosporium*, *Syncephalastrum* and *Actinomucor* were found in *L. mauritii*, unlike the other two species of earthworms. It is important to mention that the digested material for all three species came from organic waste.

Krištůfek *et al.* (1992, 1993) observed and increase in the number of bacteria, Actinomycetes and fungi in the anterior section of the digestive tract of *Lumbricus rubellus* while the opposite occurred in *Aporrectodea caliginosa* and *A. caliginosa*.

The Actinomycete community present in the intestine of *Eisenia fetida*, mainly *Streptomyces caeruleus*, develops better in the intestines in comparison to soil and helps the earthworms to metabolize organic matter and decomposition of substances from plant origin (Polyanskaya *et al.*, 1996).

BACTERIAL DIVERSITY IN THE DIGESTIVE TRACT OF EARTHWORMS

Soil is the key system in the functioning of terrestrial ecosystems. Vital processes take place within this system: decomposition and nutrient flow (Bashan *et al.*, 2004) Biological activities control theses processes, among them, prokaryotes and earthworms (Table 3) (Davidson and Stahl, 2006; Fragoso *et al.*, 2001).

Jolly *et al.* (1993) demonstrated the existence of physical contact between some filamentous, segmented bacteria and intestinal mucus of the species *Octolasion lacteum* and *Lumbricus terrestris*. The results showed bacterial filaments joined to the intestinal walls of the earthworms by means of hook structures. Therefore, it is concluded that the bacteria may be adapted to live within the intestines of the earthworms. Thus diverse studies on the main bacteria within the intestinal walls of earthworms have been conducted.

An observation was made for the specie *Eisenia fetida* coming from contaminated soil in an industrial zone. There was an increase in 91 colonies, further divided into 12 groups: *Aeromonas* 6%, *Agromyces* 3%, *Bacillus* 31%, *Bosea* 1%, *Gordonia* 6%, *Klebsiella* 6%, *Microbacterium* 7%, *Nocardia* 2%, *Pseudomonas* 10%, *Rhodococcus* 19%,

Table 3: Bacterial diversity identified within the intestinal walls of earthworms

Earthworm	Habitat	Classification	Bacteria	References
Onychochaeta boricana	Soils por in organic matter	Growth promoting bacteria	*Bacillus* sp.	Valle-Molinares *et al.* (2007)
Onychochaeta boricana	Forest soils	Growth promoting bacteria	*Bacillus* sp.	Mendez *et al.* (2002)
Eisenia fetida	Soils from industrial zones	Growth promoting bacteria	*Klebsiella* sp.	Hyun-Jung *et al.* (2004)
Lumbricuss rubellus	Agroecosystems	Growth promoting bacteria	*Azotobacter* sp. *Enterobacter* sp. *Pseudomonas* sp. *Klebsiella* sp. *Azotobacter* sp. *Pseudomonas* sp.	Singleton *et al.* (2003)
Onychochaeta boricana	Soils por in organic matter	Biocides	*Bacillus thuringieusis*	Valle-Molinares *et al.* (2007)
Eisenia fetida	Soils from industrial zones	Other bacteria	*Flavobacterium* sp. *Nocardia* sp. *Gordonia* sp. *Vibrio comma* *Clostridium welchii* *Proteus vulgaris* *Serratia marcesceus* *Mycobacterium* sp.	Hyun-Jung *et al.* (2004)

Tsukamurella and *Streptomyces* 7%. The genus *Bacillus* was the dominant group found in the intestines of the earthworm (Hyun-Jung *et al*., 2004).

The specie *Eisenia fetida* may establish a symbiotic relationship with bacteria from the genus *Acidovorax,* given that these bacteria form nodules in the ampules of the nephridium of the earthworms and help in the process of protein decomposition (Davidson and Stahl, 2006).

On the other hand, Valle-Molinares *et al*. (2007) identified seven species of bacteria from the genus *Bacillus*: (*B. insolitus*, *B. megaterium*, *B. brevis*, *B. pasteurii*, *B. sphaericus*, *B. thuringiensis* and *B. pabuli*) within the intestines of *Onychochaeta borincana.* All these species are typical soil bacteria. In addition, it was found that the microbial weight of the intestinal region decreased from the anterior to posterior section. Additionally, it was observed that some bacteria increased in the posterior section of the intestines, maybe because for a many bacteria this portion presents adequate conditions for their development.

Mendez *et al*. (2003) indicated that the bacteria can accomplish a type of mutualism during their passage through the digestive tracts of earthworms, which have not yet been studied in other genuses of bacteria. The existence of a mutual symbiotic relation between *O. boricana* and *B. cereus* has been proposed.

MOLECULAR TECHNIQUES USED IN THE STUDY OF BACTERIAL DIVERSITY WITHIN THE DIGESTIVE TRACTS OF EARTHWORMS

The use of molecular techniques has manifested the unawareness of biological diversity, systemic classification and taxonomy given that the majority of microorganisms are not cultivatable in conventional mediums and also the analysis of functional genes key in important soil processes such as denitrification, nitrification nitrogen fixation and methane oxidation (Nogales, 2005).

Table 4 shows some molecular techniques used in the identification of bacteria found within the digestive tracts of earthworm, applying basic microbiological culture techniques (Santiago, 1995). Many forms of microscopy (Kryštůfek *et al*., 1994) and molecular biology (Hyun-Jung *et al*., 2004; Singleton *et al*., 2003) (with the last being currently one of the most used techniques) and its application in soil microbiology studies mainly in the digestive tracts of earthworms, represent a great advance in the knowledge of different ecosystems.

The study of the diversity of microorganism is currently based on protein analysis, DNA or RNA of the ribosomal genes 16S or 23S and the presence of enzymes or enzyme alleles (Curry and Schmidt, 2007).

The identification process of bacteria associated with the intestines of earthworms is difficult if these microorganisms require growing conditions present only in the intestines of the earthworms. Singleton *et al*. (2003) used molecular techniques such as Polymerase Chain Reaction (PCR), for identifying bacteria associated with the intestines of *Lumbricus rubellus*, which were absent from the earthworms skins.

Fluorescent microscopy has been used for studying population variations of the bacteria within each intestinal segment of the Oligochaetes.

Fischer *et al*. (1995, 1997) studied the digestive intestinal walls of *O. borincana* using trace electronic microscopy and found bacteria from the genus *Bacillus* adhered to the intestine of this oligochaete being abundant in the frontal area and less abundant in the posterior parts. The researchers suggested the smooth surface of the posterior parte offers less possibility of adhesion for the bacteria on the intestinal wall but is appears that they may have found a favorable environment in the posterior part.

CONCLUSIONS

The importance of microbial diversity using conventional and molecular techniques is still far from understanding the role of the microorganisms within the digestive tracts of earthworms and within the soil in the functioning of ecosystem, particularly in those which have not being laboratory grown and for those whose metabolic capacities is totally unknown. They are key in important soil processes such as denitrification, nitrification, nitrogen fixation, methane oxidation, growth hormone production, phosphorous solubilizers and control of microbial pathogens.

Table 4: Molecular techniques used in the identification of bacterial diversity within the digestive tract of earthworms

Earthworm	Technique	Bacteria	References
Onychochaeta borincana	Electron microscopy and PCR	Identified the genus *Bacillus* with seven different species and β-*bacteria hemolitica*	Valle-Molinares *et al*. (2007)
Eisenia fetida	PCR-16sDNA	Identified 22 bacteria	Hyun-Jung *et al*. (2004)
Onychochaeta borincana	Electron microscopy	Identified seven bacteria of the genus *Bacillus* sp.	Méndez *et al*. (2003)
Lumbricus rubellus	Fluorescent *in situ* Hybridation techniques and 16sRNA	Identified: *Acidobacteria*. *Paenibacillus*, *Pseudomonas* sp., *Actinobacteria*	Singleton *et al*. (2003)
Lumbricus rubellus	*In situ* hybridation technique	*Bacillus megaterium* within the digestive tract	Fischer *et al*. (1995)

The bacterial diversity within the digestive tracts of earthworms from different genuses and ecotypes presents a variety of geniuses and prokaryote species, attributed to their habitat, soil type, climate, substrate type and biota.

The earthworm is an incubator and disperser of bacteria of agricultural importance given that some species found, produced microbial control of certain pathogens (*Burkholderia*, *Enterobacter*, *Agrobacterium Serratia, Pseudomonas*), plant growth promoters through the production of the indole-3-acetic acid (*Klebsiella*, *Azotobacte*, *Aeromonas*, *Bacillus*), produced siderophore (*Pseudomonas*, *Bacillus*, *Proteus*) and phosphate solubilizers unavailable to plants (*Bacillus*, *Enterobacter*, *Erwinia*, *Pseudomonas*, *Rhizobium*, *Serratias*, *Agrobacterium*, *Burkholderia*, *Achromobacter*, *Microccocus*, *Aerobacter*, *Flavobacterium* and *Erwinia*).

The genetic characterization of the isolated bacteria allows us to broaden our knowledge of the bacterial diversity within the different structures or parts of the digestive tracts and under different natural ecosystems presenting anthropogenic activities and the study of plant effect.

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