



## GUT BACTERIAL LOAD ANALYSIS OF EARTHWORMS (*Eudrilus eugeniae*) – A CONTROLLED LABORATORY STUDY

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### ABSTRACT

Earthworms are unique members of the soil vicinity. They maintain the soil fertility health. However, their interactions with soil microorganisms are till date not good understands. The present research was carried out to identify the microbial analysis of earthworm gut. The *Eudrilus eugeniae* was selected as a laboratory tool for this research. A bacterium was observed throughout the gut. *Pseudomonas sp*, *Strepto coccus sp*, *Bacillus subtilis* and *Bacillus cereus* was observed in our laboratory tool (*Eudrilus eugeniae*).

**Keywords:** Earthworm; Gut; Microbes.

### INTRODUCTION

Earthworm gut is tubular structure extending from mouth to the anus; its different regions are the muscular pharynx, oesophagous, intestine and associated digestive glands. The gut contains usually comprise mucus, organic and mineral matter. An analysis of gut contents in earthworm revealed the occurrence of different kinds of symbiotic like microfungi, bacteria, protozoa, etc [1]. The earthworm gut environment is anoxic, pH 6.9 with about 50% water content. The gut bacteria are enriched in total carbon, organic carbon and total nitrogen with a carbon to nitrogen ratio of 7 [2]. Gut of earthworm may be considered as ideal habitats for bacteria [3], because earthworms can directly regulate microbial population of their gut by consuming large amount of soil. Microorganisms are act as a vital food component of soil invertebrates including earthworms [4]. The microorganisms available in the gut of earthworm

species are mostly related to the soil micro flora. If microorganisms were to act as symbionts in digestion by this earthworm, they would probably be associated with its gut [5]. The aim of this research is to find out the gut microbial load of earthworm and relate our result with symbiotic relation of earthworm and microorganisms.

### MATERIALS AND METHODS

#### Experimental Animal

Earthworm species namely, *Eudrilus eugeniae* was used as a laboratory tool in current research. *E. eugeniae* was procured from Shakthi Vermicompost Farm in Vadipatti, Madurai district, Tamilnadu, India and kept on mother culture tank at Venture Institute, Madurai that was moist with de-ionized water with regular interval. The cow dung was added in the soil to enrich the medium. Mother tank was placed in a dark room at 25±2°C and 80% humidity for an acclimatization period of 2 weeks. During the acclimatization period regularly cow dung was added because it was a food for earthworms.

#### Systematic position

##### *Eudrilus eugeniae*

*Eudrilus eugeniae* was selected as the laboratory tool because it grows well at a temperature of more than 25°C but best at 30°C [6]. It is locally available in and around the South Tamilnadu.

Phylum – Annelida

Class – Oligochaeta

Order – Haplotaxida

Family – Eudrilidae



Genus – *Eudrilus*  
Species - *eugeniae*

### **Earthworm gut fluid**

The *E. eugeniae* was fed with sterile sand to clean up the digestive tracts using Whatman filter paper. The earthworms were killed in 4% paraformaldehyde and dissected from the ventral side [7].

### **Identification and Characterization of the efficient earthworm gut bacteria**

#### **Morphological Characterization**

Morphological characteristics such as abundance of growth, pigmentation, optical characteristics, size and shape were studied on nutrient agar plates.

#### **Gram's staining**

The isolate was smeared on the glass slide and heat fixed. The crystal violet dye was added, kept for one minute and washed in running tap water. Then the gram's iodine was added, kept for one minute and washed in running tap water. It was decolorized with ethanol and then counter stained with safranin and washed in running tap water. It was observed in compound microscope. The bacteria that retained the crystal violet stain (appear violet) was designated as gram positive ( $G^+$ ) and those cells that stained with pink colour are called gram negative ( $G^-$ ).

#### **Spore Staining (Schaeffer-Fulton Method)**

The isolate was smeared in the slide and heat fixed. Then the slide was flooded with malachite green and steam heat the slide for 2-3 minutes. Cool the slide and washed in running tap water. It was then added with counter stain safranin and kept for 30 seconds. Wash the slide with running tap water. Air dried the slide and examined under oil immersion, in microscope. The spores appeared green in colour while the vegetative cells appeared red in colour.

#### **Biochemical Characterization**

##### **Indole Production Test**

Indole production test is used to test whether the organism can have the ability to produce indole. Peptone broth was prepared, sterilized and cooled. Inoculate the test organism to the sterile peptone broth and incubate the tubes at 37°C for 24 hrs. The culture producing the cherry red colour ring following the addition of Kovac's reagent indicated as positive. The absence of red colouration indicated a negative result.

##### **Methyl Red Test**

Methyl red test is employed to detect the ability of microorganisms to oxidize glucose with the production

of high concentration of acid end products. The isolated organisms were inoculated into test tubes containing sterile MR-VP broth and incubate the tubes for 24 to 48 hrs 37°C. After incubation, add 7-8 drops of methyl red indicator and appearance of red colour indicated the positive result.

##### **Voges-Proskauer Test**

This test is also known as the acetoin production test. This test is used to differentiate the capacity of organisms to produce some nonacidic (or) neutral end product such as acetyl methyl carbinol (or) 2,3-butanediol. The isolated organisms were inoculated into sterile MR-VP broth tubes and incubate for 24 hrs at 37°C. Development of deep rose colour following the addition of Barritt's reagent A and B indicated the positive result. The absence of deep rose colour is a negative result.

##### **Citrate Utilization Test**

Some of the organisms were capable of utilizing citrate as the sole carbon source and mono ammonium phosphate as the sole source of nitrogen. As a result, the pH of the medium changes, this was indicated by changes in the indicator present in the medium. Simmon's citrate medium was prepared, sterilized and kept in a slanting position and allowed the tubes to solidify. The test organisms were streaked on the slant and incubated at 37°C for 24 hrs. The change of color from green to Prussian blue coloured slant indicated the positive result.

##### **Triple Sugar Iron Test**

TSI test is used to differentiate the isolate according to the ability to ferment lactose, sucrose and glucose and production of hydrogen sulfide. Triple sugar iron agar medium was prepared and sterilized. Kept the tubes as slant and butt and allow it to solidify. Streak a loop full of test organisms on the surface of the slant and incubate at 37°C for 24 hrs. Acidification of the medium caused by the isolates attacking one of the sugars causes the phenol red indicator to change to yellow colour. Gas production is indicated by bubble formation in the butt. Hydrogen sulphide production causes the formation of a black precipitate at the junction between the slope and the butt.

##### **Nitrate Reduction Test**

This test is used to detect whether the organisms reduced the nitrates to nitrites or not. Nitrate broth was prepared and sterilized. Inoculate one loop full of test culture and incubate at 37°C for 96 hrs. Following incubation, add 0.1 ml of test reagent (Sulphanilic acid and  $\alpha$ -naphthalamine) to the test culture. A red colour developing within a few minutes the presence of nitrites and hence the ability of the organisms to reduce nitrates.



## RESULTS

**Table 1. Biochemical characterization of bacteria isolated from gut tissues of earthworm**

Content	EG1	EG2	EG3	EG5
Gram Staining	G <sup>-</sup>	G <sup>+</sup>	G <sup>+</sup>	G <sup>+</sup>
Spore Staining	Absent	Absent	Present	Present
Peptone	-	-	-	-
Methyl Red Test	-	-	-	+
Voges Prokauer	-	-	-	-
Simmon Citrate Agar	-	+	-	+
Triple Sugar Iron Agar	+	Alkaline slant	Acid (butt & slant)	Alkaline slant
Lactose fermentation	Acid with gas	-		
Mannitol		-	Acid	-
Mac Conkey	No growth	No growth		

EG-Earthworm gut

**Table 2. The Bacteria isolated in this work**

Different areas of gut tissue	Bacteria
EG1	<i>Pseudomonas</i>
EG2	<i>Strepto coccus sp</i>
EG3	<i>Bacillus subtilus</i>
EG5	<i>Bacillus cereus</i>

## DISCUSSION

Earthworms are globally accepted as ecosystem engineer by soil scientists. Earthworms, the only solution for easily recycle the municipal solid waste (MSW) into vermicompost. But in the time of vermicomposting the earthworms are combined with their gut microbes in addition to that soil microbes also interact with earthworms. Table 1 shows the biochemical results of bacteria isolated from gut tissues of earthworm (*Eudrilus eugeniae*). The bacterial population was identified out in the gut as shown in Table 2. From the table, it is clear that *Pseudomonas sp*, *Strepto coccus sp*, *Bacillus subtilus* and *Bacillus cereus* was present in the gut of *Eudrilus eugeniae*.

### Earthworms and Microorganisms

Previous authors [1] says gut contents in earthworm has different kinds of symbiotic like microfungi, bacteria, protozoa, etc. The earthworm gut vicinity may act as a selective filter as well as fermenter for soil microorganisms [8]. Earthworms can increase microbial activities by providing in their gut mucus consisting of energetic and easily metabolizable compounds [9] and considerable physico-chemical conditions: neutral pH, high moisture and ideal temperature conditions [10]. Earthworms accelerate the growth of beneficial decomposer microbes (bacteria, actinomycetes and fungi) in waste biomass [11].

The presence of gut wall bacteria of earthworms observed in this research was also reported by other workers in various earthworm species on different occasions. Due to our research we identify 4 species of bacterial stains. Out of four *Strepto coccus sp* is one of the

bacteria. Previous authors [12] find out *Rhodococcus sp* from Indian earthworm *Metaphire posthuma*. Our finding says earthworms and microorganisms have the perfect relationship to each other. Similarly, Previous authors [13] says that, earthworm gut which is described as little bacterial factory. They devour on microbes and excrete them out (many time more in number than they ingest) in soil along with nutrients nitrogen (N) and phosphorus (P) in their excreta. The nutrients N and P are further used by the microbes for multiplication and vigorous action.

Previous authors [14] suggested in the first time he done research in microbiology of earthworm gut, researchers have attempted to study earthworm gut microbes using direct culture methods (Eg: 15; 16) and electron microscopy [17]. Previous authors [17] studied the digestive tube of earthworm by Scanning Electron Microscopy (SEM). They find the cocoid bodies from the foregut of *O. cyaneum*. In *O. borincana* they were only seen in the hindgut.

In our research we find out the two species of *Bacillus* (*Bacillus cereus* & *Bacillus subtilus*) in the gut of *E. eugeniae*. Similarly, [18] identified the following seven species of bacteria from the genus *Bacillus* (*B. insolitus*, *B. megaterium*, *B. breris*, *B. pasteurii*, *B. sphaericus*, *B. thuringiensis* and *B. pabuli*) within the intestine of *Onychochaeta borincana*. All these species are typical soil bacteria. Our research agrees with previously [19] reported that there was an increase in colony counts of *Bacillus sp* and find out seven different species of *Bacillus* from the gut of *O. borincana*. According to previous authors [20] the *Bacillus subtilus* was the



predominant one in earthworm gut. This statement supports our research.

For studies on bacterial species within the intestinal tract of earthworms, diverse methods and techniques have been used which have helped in identifying species of the genus *Bacillus*, *Pseudomonas*, *Klebsiella*, *Azotobacter*, *Serratia*, *Aeromonas* and *Enterobacter* [18; 8; 21].

We find out the *Strepto coccus sp* in the gut of *E. eugeniae*. On the other hand, Previous authors [22] analyzed the earthworm gut and find out the presence of cocci, rod-shaped bacteria and filamentous microorganisms in *Lumbricus terrestris* L., 1758 and *Octolasion cyaneum*. *Lumbricus terrestris* and *Octolasion cyaneum* have only two rod shaped organisms in their gut. In contrast, the Transmission Electron Microscopy (TEM) investigation of *L. terrestris* hindgut showed several rod-shaped bacteria over a relatively small area [17]. Previous authors [23] concluded that the earthworm intestinal tract may have a major impact on the composition of the soil bacterial community and so gut passage should be taken into consideration when assessing the risk of releasing non-indigenous, Eg: genetically engineered, bacteria into terrestrial ecosystems. It was proposed that earthworms derive more of its energy and nutrients from gut specific microbiota than from microbiota already present in the ingested soil [24].

Previous authors [25] isolated the eight bacterial groups from fresh soil and gastrointestinal tract of the earthworms *L. terrestris* and *Aporrectodea caliginosa* were reported by single strand conformation polymorphism (SSCP) analysis using both newly designed 16S rRNA gene specific primer sets targeting Alphaproteo bacteria, Betaproteo bacteria, Gammaproteo bacteria, Deltaproteo bacteria, Bacteroides, Verrucomicrobia, Planctomycetes and Firmicutes. Previous authors [26] found the gut wall associated bacterial communities from *Lumbricus terrestris*, *L. friend*, *Aporrectodea caliginosa* and *A. longa*. The abundance of specific gut wall associated bacteria, including proteobacteria, firmicutes and an actinobacterium was dependent on the ecological group.

The bacterial counts in guts was higher than the surrounding soil ecosystem [27-32] and as the organic matter ingested passes through the gut, it undergoes biochemical changes effected by gut-inhibiting bacteria [31]. [33] who found number of microorganisms (bacteria, fungi) in alimentary tract earthworms were six times higher in comparison with the surrounding soil. Microbes of the earthworm gut are higher than those in surrounding soil and not methane emission, occurs in the gut of *Aporrectodea caliginosa* and *Lumbricus rubellus* [34].

Some previous studies proposed that the earthworm gut microbial community is qualitatively not much different from the microbial community in the

surrounding soil [14], later studies found significant differences for selected phylogenetic groups or functional guilds of microorganisms, Eg: Proteobacteria [35], Actinobacteria, denitrifiers or cellobiose utilizers [36; 37].

### Food of Earthworm

Microorganisms may constitute a very important part of the diet of earthworms, which can feed on them selectively [38; 39]. Fungi and bacteria are assumed to be the main source of food for earthworms [40]. While feeding, earthworms regulate the growth of soil microorganisms by eating some microbial populations and providing ideal conditions for the growth of others in their digestive tract and in casts [41]. The bacterial sp profiles are very closely similar in soil and in the gut contents of many earthworm species, suggesting an absence in the gut of any indigenous bacterial groups [14]. Earthworms are ubiquitous that ingest large amounts of mineral soil and organic matter containing a variety of microorganisms [42]. Earthworms can directly balance the microbial population (Bacteria and Fungi) by consuming large amount of soil [41, 43].

### Interactions between earthworm and soil microorganisms

However, poor understood on the interactions between earthworm and soil microorganisms. A beneficial effect of earthworm activity on soil properties is contributed to interactions with soil ecosystem microorganisms [44]. Earthworms and microbes act symbiotically and synergistically to increase the decomposition of the organic matter in the waste. It is the microorganisms helpful to break down the cellulose in the food waste, grass clippings and the leaves from garden wastes [45]. Interactions between earthworms and soil microorganisms are important factor for soil processes such as decomposition and transformation of plant residue, humus formation and the formation of the pool of nutrient elements and microbial communities. The wide spectrum of these interactions makes it possible to speak about a close relationship between earthworms and microorganisms [40]. The gut of many soil organisms contains microbial communities that usually helpful in the digestion. These microbial-animal relationships create mutualisms. Earthworms are also having a mutualistic relationship with soil microorganisms (Bacteria, Fungi, etc) passing through their digestive tract, but the nature and role of the microbiota inhabiting their gut are virtually unknown.

### CONCLUSION

In conclusion, we would like to conclude that the earthworm gut is ideal vicinity for microorganisms such as bacteria, etc. These finding suggest that the earthworm gut favorable for the growth and activity of certain bacterial species. Understanding the world of earthworm





gut wall associated bacterial community 100% helpful for successive agriculture and recycle of municipal solid waste into compost without any side effects. We have identified that different Gram negative and Gram positive bacteria in gut of *E. eugeniae*. Further research is needed for understanding the earthworm gut microflora.

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