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# MICROBIAL ANALYSIS OF THE GUT OF SELECTED EARTHWORMS
## *(Eisenia foetida, Eudrilus eugineae, Perionyx excavates)*

A dissertation submitted to Scott Christian College (Autonomous),

Affiliated to MANONMANIAM SUNDARANAR UNIVERSITY,

THIRUNELVELI .

In partial fulfilment of the requirements for the award of the
Degree of Master of Science in Microbiology

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# CERTIFICATE

This is to certify that the project work **"MICROBIAL ANALYSIS OF THE GUT OF SELECTED EARTHWORMS *(Eisenia foetida, Eudrilus eugineae, Perionyx excavates)"*** is a bonafide record of work done by **G.VISHNU, Reg .No.150252** during the acadamic year 2015-2017, submitted for the practical fulfilment of the requirements for the award of the degree of **"MASTER OF SCIENCE IN MICROBIOLOGY",** to Scott Christian College (Autonomous) Nagercoil, affiliated to Manonmanium Sundaranar University, Thirunelveli. It is further certified that this work has not formed for the award of any Degree or Diploma or other similarities of any other university

**Head of the department** **Signature of the guide**

Project submitted to the viva voce examination held on

Examiners: 1.

2.

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# DECLARATION

I here by declare that this project report entitled **"MICROBIAL ANALYSIS OF THE GUT OF SELECTED EARTHWORMS *(Eisenia foetida, Eudrilus eugineae, Perionyx excavates)"*** Submitted to Scott Christian Collage (Autonomus) Nagercoil, affiliated to **Manonmaniam Sundaranar University,** Tirunelveli, in March 2017. This is an original and independent work for the award of Degree of Master of Science in Microbiology, under the guidance of **Mrs. K. Jenitha.**

Place : Nagercoil G.VISHNU
Date :

## ACKNOWLEDGEMENT

I have taken effoerts in this project. However it would not have been possible without the kind support and help of many individuals. I would like to extend my sincere thanks to all of them.

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My thanks and appreciations also go to my colleague in developing the project and people who have willingly helped me out with their abilities.

**G.VISHNU**

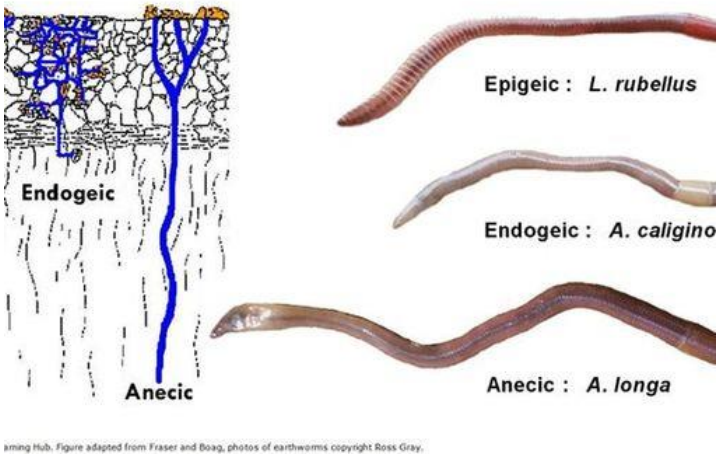
# 1. INTRODUCTION

Vermicomposting is the process by which earthworms are used to convert organic materials, usually wastes, into a humus-like material known as vermicompost. The term vermitechnology is used in a general sense to refer to the utilization of surface and subsurface varieties of earthworms in composting and management of soil. Vermicast is the faecal matter released by the earthworms. The term vermicast is also used in a general sense to mean the product of vermicomposting, the vermicompost. Vermicomposting is a non thermophilic biological oxidation process in which organic materials are converted into vermicompost which is a peat like material, exhibiting high porosity, aeration, drainage, water holding capacity and rich microbial activities, through the interactions between earthworms and associated microbes (Arancon *et al*., 2004). Vermiculture is a cost-effective tool for environmentally sound waste management (Asha *et al*., 2008)

Earthworms are capable of transforming garbage into 'gold'. Charles Darwin described earthworms as the 'unheralded soldiers of mankind', and Aristotle called them as the 'intestine of earth', as they could digest a wide variety of organic materials. Earthworms play an important role in carbon turnover, soil formation, participates in cellulose degradation and humus accumulation. Earthworm actively profoundly affects the physical, chemical and biological properties of soil. Earthworm's intestine contains a wide range of microorganisms, enzymes and hormones which aid in rapid decomposition of half digested material transforming them into vermicompost in a short time (nearly 4-8 weeks) (Nagavallema *et al*., 2004) compared to traditional composting process which takes the advantage of microbes aloneand thereby requires a prolonged period (nearly 20 weeks) for compost production (Sanchez Monedero *et al*., 2001).

Earthworms are classified into epigeic, anecic and endogeic species based on definite ecological and trophic functions. Epigeic earthworms are smaller in size, with uniformly pigmented body, short life cycle, high reproduction rate and regeneration. They dwell in superficial soil surface within litters, feeds on surface litter and mineralize them. They contain an active gizzard which aids in rapid conversion of organic matter into vermicomposts. Epigeic earthworm includes *Eisenia fetida*, *Lumbricus rubellus*, *Bimastus minusculus*, *Dendrodrilus rubidus*, etc. Endogeic earthworms are small to large sized worms, with weakly pigmented body, life cycle of medium duration, moderately tolerant to disturbance, forms extensive horizontal burrows and they are

geophagous feeding on particulate organic matter and soil. They can efficiently utilize energy from poor soils, hence can be used for soil improvements. Endogeics include *Aporrectodea caliginosa*, *Octolasion cyaneum*, *Dontoscolex corethrurus*, etc. Aneceic earthworms are larger dorsally pigmented worms with low reproductive rate, sensitive to disturbance, nocturnal, phytogeophagous, bury the surface litter, forms middens and extensive deep, permanent vertical burrows and live in them. *Lumbricus terrestris*, *Lumbricus polyphemus* and *Aporrectodea longa* are examples of aneceic earthworms (Kooch and Jalilvand, 2008). Epigeics and aneceics are harnessed largely for vermicomposting (Asha *et al*., 2008). Epigeics namely *Perionyx excavates* (Suthar and singh, 2008) and *Eisenia anderi* (Munnoli *et al*., 2010) have been used in converting organic wastes into vermicompost. Earthworms thus act as natural bioreactors, altering the nature of the organic waste by fragmenting them.




arming Hub. Figure adapted from Fraser and Boag, photos of earthworms copyright Ross Gray.

**Fig 1. TYPES OF EARTHWORMS**

Earthworm's gut is a straight tube starting from mouth followed by a muscular pharynx, oesophagus, thin walled crop, muscular gizzard, foregut, midgut, hindgut, associated digestive glands and ending with anus. The gut is an effective tubular bioreactor, which maintains a stable temperature regulatory mechanism, thus accelerating the rates of the bioproceses and preventing enzyme inactivation caused by high temperatures. The gut consisted of mucus containing protein and polysaccharides, organic and mineral matters, amino acids and microbial symbionts viz., bacteria, protozoa and microfungi. The increased organic carbon, total organic carbon and nitrogen moisture content in the earthworm gut provide an optimal environment for the activation of dormant microbes and germination of endospores, etc. A wide array of digestive enzymes such as amylase, cellulase, protease, lipase, chitinase and urease were reported from earthworm's alimentary canal.

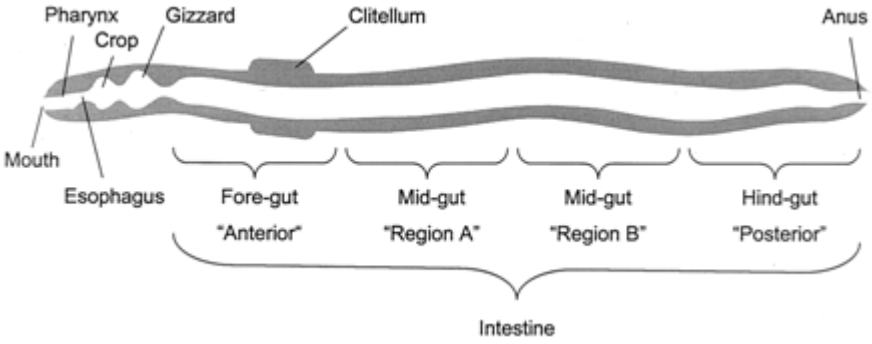



**Fig 2. GUT OF EARTHWORM**

Enzyme activity in earthworms is regionally specialized and influenced by physiological state, age and microorganisms. Digestive enzymes like cellulase, xylanase, acid phosphatase and alkaline phosphates were found to be more in the gut of *Eisenia fetida* as compared to *Eudrilus eugeniae*. Amylase, cellulose, acid phoshatase, alkaline phosphates and nitrate redutase were secreted in the gut of the earthworms due to increase in presence of microorganisms in it. Amylase, cellulase, xylanase, endoglucanase, cellobiase, acid phosphatase, alkaline phosphate and nitrate reductase produced jointly by earthworms and gut microflora are supposed to play a central role in the process of digestion and humification of soil organic matter. Amylase, cellulase, xylanase,

endoglucanase and cellobiase act upon the complex biomolecules such as starch, cellulose, xylan and cellodextrins. Acid phosphatase and alkaline phosphates and nitrate reductase are involved in the metabolism of phosphates and nitrogen. The gut microbes were found to be responsible for cellulose and mannose activities (Munnoli *et al.*, 2010). As the organic matter passes through the gizzard of the earthworm it is grounded into a fine powder after which the digestive enzymes, microorganisms and other fermenting substances act on them further aiding their breakdown within the gut, and finally passes out in the form of "casts" which are later acted upon by earthworm gut associated microbes converting them into manure product, the "vermicompost" (Dominguez and Edwards, 2004). When the organic matter passes through the gut of the earthworm, it gets mixed up with the digestive enzymes and finally leaves the gut in partially digested form as "casts" after which the microbes takes up the process of decomposition contributing to the maturation phase (Lazcano *et al.*, 2008).

The gut of the earthworm constitutes a unique microenvironment in soils. The selective digestion of microbes in the gut influences the type of nutrients that are available for subsequent assimilation by both the earthworm and members of the gut microflora. The variation in the microbial population in the earthworm's gut maybe because of their nutritional needs and digesting ability of the earthworms. The bacteria in the foregut helps to digest the food particles, actinomycetes in the midgut helps to destroy the pathogens by antagonistic activity and the fungi helps to bind the waste particles as casting in the hindgut. We look forward to work on the types of microbes present in the gut and the enzymes produced by them extracellularly that in turn helps in the digestion of the waste products into valuable castings or vermicompost.

# 2.AIM AND OBJECTIVES

## 2.1. Aim

To screen the microbiology in the gut of earthworms ( *Eisenia foetida, Eudrilus eugineae and Perionyx excavates*) which are used in the management of biowaste.

## 2.2.Objective

- Dissection of selected earthworms
- Enumeration and identification of Heterotrophic bacteria from the gut of selected earthworms.
- Enumeration and identification of Fungi from the gut of selected erthworms.

## 3. REVIEW OF LITERATURE

Charles Darwin recognized and described the importance of earthworm activity in soils. Earthworms belonging to class Oligochaeta comprise approximately 800 genera and 8000 species that account for up to 90% of invertebrate biomass present in soil. The successful management and exploitation of earthworm bioresources has the potential to deliver significant economic and environmental benefits, especially in light of global concerns regarding sustainable land use, food security and climate change. They are ubiquitous, abundant and highly productive organisms; they are 'keystone species' in soil food webs and 'ecosystem engineers' in soils (Brown *et al*., 2000). Effects of vermicomposting on pH, electrical conductivity (EC), C:N ratio and other nutrients have been documented. Earthworm activity reduced pH and C:N ratio in manure (Atiyeh *et al*., 2000). The observed increase of total phosphorous (TP) in vermicompost is probably due to mineralization and mobilization of phosphorus resulting from the enhanced phosphatase activity by microorganisms in the gut epithelium of the earthworms (Zhang *et al*., 2000). The microbial composition of earthworm intestine contents has been considered to reflect that of the soil and ingested plant remains, but there is evidence of the possible existence of an indogenous, autochthonous gut flora in some earthworm species (Toyota and Kimura., 2000). Bacteria present in the gut of earthworms are mainly plant growth promoters, free-living nitrogen fixers and phosphate solubilizers (Martínez-Romero., 2001). 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 (Young *et al*., 2001). Earthworms intestine contains a wide range of microorganisms, enzymes and hormones which aid in rapid decomposition of half-digested material transforming them into vermicompost in a short time compared to traditional composting process which takes the advantage of microbes alone and thereby requires a prolonged period for compost production (Sánchez-Monedero *et al*., 2001).

Earthworm possesses an immense bacterial diversity within their digestive/e tracts and is very little explored mainly because of the non-cultivatable character of a large quantity of microorganisms

which mainly come from soil. 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. Anecic earthworms have a longer gut, a simpler typhlosole with less folding, a longer gut transit time and sharper gut contractions, as compared with endogeics (Breidenbach, 2002). Earthworms promote the growth of 'beneficial decomposer bacteria' in wastewater and acts as aerators, grinders, crushers, chemical degraders, and biological stimulators (Sinha *et al*., 2002). Vermicompost addition to soils planted with tomatoes, peppers, strawberry and grapes showed a significant reduction of plant parasitic nematodes and increased the population of fungivorous and bacterivorous nematodes compared to inorganic fertilizer treated plots (Arancon *et al*., 2002). Earthworms harbor 'nitrogen-fixing' and 'decomposer microbes' in their gut and excrete them along with nutrients in their excreta (Singleton *et al*., 2003). The occurrence of gut wall bacteria of earthworms reported in this study was also observed by other workers in various earthworm species on different occasions. Members of the Firmicutes were found in the intestinal tissues of earthworm species *L.terrestris, Octolasion cyaneum, Lumbricus rubellus* and *Onychochaeta borincana* (Singleton *et al*., 2003). 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. Earthworm 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 ($NH4+$) and nitrates ($NO3^{-}$) (Desjardins *et al*., 2003).

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 keys in important soil processes such as denitrification, nitrification, nitrogen fixation, methane oxidation, growth hormone production, phosphorous solubilizers and control of microbial pathogens. 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. From an agricultural point of view, the most important family of earthworm is Lumbricidae and includes the genuses *Lumbricus, Aporrectodea, Allolophora,*

*Dendrobaena, Eisenia, Helodrilus, Octalasion* and *Eophila* (Edward., 2004). The genus Bacillus was the dominant group found in the intestines of the earthworm (Hyun-Jung *et al*., 2004). Chemical analysis showed vermicompost had a lower pH, EC, organic carbon (OC) C:N ratio, nitrogen and potassium and higher amounts of total phosphorous and micronutrients compared to the parent material (Hashemimajd *et al*., 2004). During vermicomposting the heavy metals forms complex, aggregates with humic acids and other polymerized organic fractions resulting in lower availability of heavy metals to the plant, which are otherwise phytotoxic (Dominguez and Edwards 2004). Earthworms influence primary soil functions and processes, such as soil structure formation, soil carbon dynamics and biogeochemical cycles (Brown and Doube., 2004). The microbial profile of the gut content is akin to that of soil and feed resources, it is not a coincidental combination of the microorganisms present in soil (Egert *et al*., 2004). However, based on studies conducted on insects and faunal gut-associated microbial communities; we can expect the microbial profile of the gut to be an important determinant of earthworm metabolism (Zientz *et al*., 2004). Enzymatic activity characterization and quantification has a direct correlation with type and population of microbes and reflects the dynamics of the composting process in terms of the decomposition of organic matter and nitrogen transformations and provide information about the maturity of the compost (Tiquia 2005). Bacteria with homology to *Geobacter sulfurreducens* and *Rhodococcus* sp. were more abundant (relative to other bacteria) in the gut walls of endogeic as compared with anecic species and this might reflect the ability of endogeics to use more complex stabilized soil humic substances than do anecic species (Briones *et al*., 2005).

All bacteria found within earthworms were also detected within the associated soil samples. This fact and the nature of this study means that it is not possible to determine whether bacteria tightly associated with the gut wall share a symbiotic or a mutualistic metabolic relationship with their host. Bacteria may be selected from the ingested material because they confer the host with a metabolic advantage (for example vitamins, minerals, digestive enzymes) and they could form an opportunistic association with the gut wall. Alternatively, some gut wall bacteria may represent true symbionts that form stable populations and have a critical function in host nutrition by enhancing metabolite acquisition, synthesis or catabolism (Moran, 2006). Acidovorax bacteria are well-known nephridial symbionts of many earthworm species, and it was postulated that they could be important in protein degradation during nitrogenous excretion by earthworms (Davidson

and Stahl., 2006). Verma *et al*., (2006) isolated a chlorinated hydrocarbon-degrading *Rhodococcus* species from the gut of an Indian earthworm, Metaphire posthuma. Changes in soil structure and C sequestration can significantly alter the soil biological functions and hence affect organic matter decomposition (Byers *et al*., 2006). The importance of habitat in the formation of gut wall-associated bacterial communities within and across species supports the hypothesis that the acquisition of a new diet is a fundamental driver for the evolution of new species (Moran, 2006). Earthworms intervene in soil biological regulation systems, possesses the capacity to remove soil particles and produce organomineral structures called biogenic structures (Rossi *et al*., 2006). 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). 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*. 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). 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). Physical, physiological and biochemical properties dictate the metabolic capacity of the earthworm gut (Drake and Horn, 2007).

Earthworm activity engineers the soil by forming extensive burrows which loosen the soil and makes it porous. These pores improve aeration, water absorption, drainage and easy root penetration. Soil aggregates formed by earthworms and associated microbes, in the casts and burrow walls play an indispensible role in soil air ecosystem. These aggregates are mineral granules bonded in a way to resist erosion and to avoid soil compaction both in wet and dry condition. Earthworms speed up soil reclamation and make them productive by restoring beneficial microflora. Thus degraded unproductive soils and land degraded by mining could be engineered physically, chemically and biologically and made productive by earthworms. Hence earthworms are termed as ecosystem engineers. Comparison of compost and vermicompost showed that vermicompost had significantly less C:N ratios as they underwent intense decomposition (Lazcano

*et al*., 2008). Earthworms body acts as a 'biofilter' and remove the biological oxygen demand (BOD), chemical oxygen demand (COD), total dissolved solids (TDS) and total suspended solids (TSS) from wastewater by 90%, 80–90%, 90–92% and 90–95% respectively by 'ingestion' and biodegradation of organic wastes, heavy metals, and solids from wastewater and by their 'absorption' through body walls (Sinha *et al*., 2008). Traditional thermophilic composts promote only selected microbes while non-thermophilic vermicomposts are rich sources of microbial diversity and activity and harbour a wide variety of antagonistic bacteria thus acts as effective biocontrol agents aiding in suppression of diseases caused by soil-borne phytopathogenic fungi (Singh *et al*., 2008). Earthworm feeding reduces the survival of plant pathogens such as *Fusarium* sp. *and Verticillium dahliae* and increases the densities of antagonistic fluorescent pseudomonads and filamentous actinomycetes while population density of *Bacilli* and *Trichoderma* spp. remains unaltered (Elmer 2009). The gut microbes of earthworm were found to be responsible for the cellulase and mannose activities (Munnoli *et al*., 2010). Earthworm activity increases the population of plant growth-promoting rhizobacteria (PGPR) (Sinha *et al*., 2010).

# 4. MATERIALS AND METHODS

## 4.1 Collection of earthworms

The specimens selected for the present investigation were the adult earthworms namely *Eisenia foetida, Eudrilus eugineae, Perionyx excavatus*. The worms were collected from Koonpura-The house of mushrooms, Trivandrum and from Vivekananda centre, Kanyakumari. Species identification was confirmed using the general characters of the worms (Table 1).

**Table 1. General characters of earthworms**

| **Features** | ***Eisenia foetida*** | ***Eudrilus eugineae*** | ***Perionyx excavatus*** |
|---|---|---|---|
| Size | 27 to 130mm × 2-6 mm | 115 to 165 mm × 4 mm | 23-120 mm × 2.5-5 mm |
| Segments | Around 100 | 160-203 | 75 to 165 |
| Colour | Banded appearance; deep purple; intersegmental grooves without pigmentation | Reddish brown dorsally and pale sandy yellow ventrally | Deep purple to reddish brown dorsally; pale ventrally |
| Behaviour | Very active; ejects yellow coelomic fluid with pungent smell | Very active | Very active; moves rapidly when disturbed |
| Prostomium | Open epilobous | Small, open epilobous | Open epilobous |
| Setae | Lumbricine; closely paired | 8 per segment | Perichaetine |

| | | | |
|---|---|---|---|
| Clitellum | Saddle-shaped; extends over six to eight segments; variable located in segments 24-34 | In 14-18; incomplete ventrally | Annular; usually in segments 14-17 |
| Spermathecal pores | Two pairs in intersegmental grooves, 9/10 and 10/11 | One pair in segment 14 | Two pairs in intersegmental grooves 7/8 and 8/9 |
| Intersegmental septa | None thickened in 14-18; incomplete ventrically | Septa between segments 4/5, 7/8/9 and 14/15 thickened | Septa 7/8 and 8/9 somewhat thick; others weak |
| Male pores | In segment 15 in raised tumescences | In segment 17 | Situated in pigment 18; closely paired |
| Female pores | As minute pores in segment 14 | In segment 14; combined with spermathecal pores | Single and median; situated in segment 14 |
| Genitial markings | Tuberculapubertatis present as solid ridges | Large central raised pad in segment 17 | None |
| Spermathecae | Usually in segments 28-30. Two pairs in segments 9 and 10 | One pair in segment 14 | Paired in segments 8 and 9 |
| Nephridia | Holoic with digitiform bladders | Holoic | Holoic |
| Cocoons | Lemon shaped | Dark coloured with tapered lemon shape | Spindle-shaped |

**Fig 3.** ***Eisenia foetida***

**Fig 4.** ***Eudrilus eugineae***

**Fig 5. *Perionyx excavatus***

**Table 2. Taxonomical classification of *Eisenia foetida***

| | |
|---|---|
| Kingdom | Animalia |
| Phylum | Annelida |
| Class | Oligochaeta |
| Subclass | Clitella |
| Order | Haplotaxia |
| Family | Lumbricodia |
| Genus | Eisenia |
| Species | *E.foetida* |

**Table 3. Taxonomical classification of *Eudrilus eugeniae***

| | |
|---|---|
| Kingdom | Animalia |
| Phylum | Annelida |
| Class | Clitellata |
| Subclass | Oligochaeta |
| Order | Haplotaxida |
| Family | Eudrilidae |
| Genus | Eudrilus |
| Species | *E.eugeniae* |

**Table 4. Taxonomical classification of *Perionyx excavatus***

| | |
|---|---|
| Kingdom | Animalia |
| Phylum | Annelida |
| Class | Clitella |
| Subclass | Oligochaeta |
| Order | Haplotaxida |
| Family | Megascolecidae |
| Genus | Perionyx |
| Species | *P.excavatus* |

### 4.2 Dissection of the selected organisms –

***Eisenia foetida, Eudrilus eugineae, Perionyx excavatus***

Healthy adults from each type was collected and allowed to starve for 24 hours. They were then disinfected with 50% ethanol. A sterile surgical blade was used for dissection. Bell pins was inserted into the ventral surface of the clittelar region and with the body slightly raised up. With the sterile surgical blade an incision was made longitudinally along the worm. The gut was then freed from the surrounding blood vessels. With a flamed foreceps the gut section was removed. This was then transferred to saline solution (0.85% NaCl solution). Then it was homogenized for 5 minutes in a vortex. This serves as the samples for further analysis.

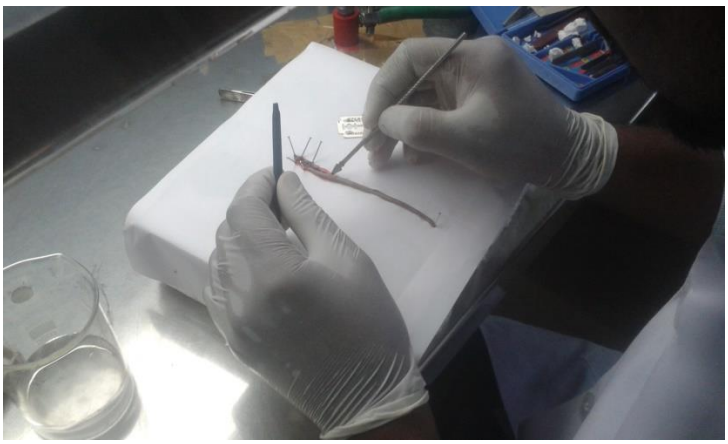


**Fig 6. Dissecting earthworm**

### 4.3 Microbial analysis of the gut of *Eisenia foetida, Eudrilus eugineae and Perionyx excavatus*

The gut isolated from the selected earthworms *Eisenia foetida, Eudrilus eugineae and Perionyx excavatus* were used for microbial analysis

### 4.4 Enumeration of Total Heterotropic bacteria

The samples were serially diluted by serial dilution agar pour plating techniques. 1.0ml of each dilutions of $10^{-4}$, $10^{-5}$ and $10^{-6}$ were plated in sterile nutrient agar media and one plate served as control. For each dilution original and duplicate plates were maintained. The plates were incubated at 37ºc for 24 hours.

### 4.5 Enumeration of Fungi

The samples were serially diluted by serial dilution agar pour plating technique. 1.0ml of each dilutions of $10^{-4}$, $10^{-5}$ and $10^{-6}$ were plated in the sterile Rose Bengal agar media and one plate served as control. For each dilution, original and duplicate plates were maintained. The plates were incubated at 28ºc for 3 days.

### 4.6 Enumeration of Actinomycetes

The samples were serially diluted by serial dilution agar pour plating techniques. 1.0ml of each of the dilution of $10^{-4}$, $10^{-5}$ and $10^{-6}$ were plated in the sterile Kenknight's agar media. One plate is served as control. For ech dilution original and duplicate plates were maintained. The plates were incubated at 28ºc 7 days.

### 4.7 Identification of Total Heterotropic bacteria from the gut of *Eisenia foetida, Eudrilus eugineae and Perionyx excavatus*

From the nutrient agar plates the isolates were identified by various morpholical and biochemical characterization tests.

| Si.No | Identification test |
|---|---|
| | **Morphological characterization** |
| 1 | Gram staining |
| 2 | Motility |
| | **Biochemical characterization** |
| 1 | Indole test |
| 2 | Methyl red test |
| 3 | Voges prouskauer test |
| 4 | Citrate utilization test |
| 5 | Triple sugar iron agar test |
| 6 | Catalase test |
| 7 | Oxidase test |
| 8 | Starch hydrolysis test |

### 4.8 Identification of fungi from the gut of *Eisenia foetida, Eudrilus eugineae and Perionyx excavatus*

From the Rose Bengal agar plates the fungal colonies were identified by colony morphology pigmenting lactophenol cotton blue mounting technique.

# 5.RESULT

## 5.1.Microbial analysis of the gut *of Eisenia foetida, Eudrilus eugineae, peroinyx excavatus*

### 5.1.1.Enumeration of heterotrophic bacteria isolated from the gut of *Eisenia foetida*

In nutrient agar plates colony count was determined both manually and also with a colony counter and the colony count (CFU/ml) was found to be tabulated (Table 6) (Plate 1A, 1B, 1C).

The number of colonies per ml of the sample was calculated by taking any one of the dilution into consideration, by using the formula,

$$\text{Number of colonies/ml of the sample} = \frac{\text{Number of colonies} \times \text{Dilution factor}}{\text{Volume of the sample plated}}$$

$$\text{Number of colonies/ml of the sample} = \frac{200.5 \times 10^4}{1}$$

$$= 200.5 \times 10^4 \text{ CFU/ml.}$$

### 5.1.2.Enumeration of fungi isolated from the gut of *Eisenia foetida*

The fungal count was determined by serial dilution spread plating technique in Rose bengal agar plates. The colony count was determined by macroscopic observation and tabulated (Table 7) (Plate 1D, 1E, 1F).

The number of colonies per ml of the sample was calculated by taking any one of the dilution into consideration, by using the formula,

$$\text{Number of colonies/ml of the sample} = \frac{\text{Number of colonies} \times \text{Dilution factor}}{\text{Volume of the sample plated}}$$

$$\text{Number of colonies/ml of the sample} = \frac{179.5 \times 10^4}{1}$$

$$= 179.5 \times 10^4 \text{CFU/ml.}$$

### 5.1.3.Enumeration of heterotrophic bacteria isolated from the gut of *Eudrilus eugineae*

In nutrient agar plates colony count was determined both manually and also with a colony counter and the colony count (CFU/ml) was found to be tabulated (Table 8)(Plate 2A, 2B, 2C).

The number of colonies per ml of the sample was calculated by taking any one of the dilution into consideration, by using the formula,

$$\text{Number of colonies/ml of the sample} = \frac{\text{Number of colonies} \times \text{Dilution factor}}{\text{Volume of the sample plated}}$$

$$\text{Number of colonies/ml of the sample} = \frac{180 \times 10^4}{1}$$

$$= 180 \times 10^4 \text{CFU/ml.}$$

### 5.1.4.Enumeration of fungi isolated the gut of *Eudrilus eugineae*

The fungal count was determined by serial dilution spread plating technique in Rose bengal agar plates. The colony count was determined by macroscopic observation and tabulated (Table 9)(Plate 2D, 2E, 2F).

The number of colonies per ml of the sample was calculated by taking any one of the dilution into consideration, by using the formula,

$$\text{Number of colonies/ml of the sample} = \frac{\text{Number of colonies} \times \text{Dilution factor}}{\text{Volume of the sample plated}}$$

$$\text{Number of colonies/ml of the sample} = \frac{189 \times 10^4}{1}$$

$$= 189 \times 10^4 \text{CFU/ml.}$$

### 5.1.5.Enumeration of heterotrophic bacteria isolated from the gut of *Perionyx excavatus*

In nutrient agar plates colony count was determined both manually and also with a colony counter and the colony count (CFU/ml) was found to be tabulated (Table 10) (Plate 3A, 3B, 3C).

The number of colonies per ml of the sample was calculated by taking any one of the dilution into consideration, by using the formula,

$$\text{Number of colonies/ml of the sample} = \frac{\text{Number of colonies} \times \text{Dilution factor}}{\text{Volume of the sample plated}}$$

$$\text{Number of colonies/ml of the sample} = \frac{247 \times 10^4}{1}$$

$$= 247.5 \times 10^4 \text{CFU/ml.}$$

### 5.1.6.Enumeration of fungi isolated from the gut of *Perionyx excavatus*

The fungal count was determined by serial dilution spread plating technique in Rose bengal agar plates. The colony count was determined by macroscopic observation and tabulated (Table 11) (Plate 3D, 3E, 3F).

The number of colonies per ml of the sample was calculated by taking any one of the dilution into consideration, by using the formula,

$$\text{Number of colonies/ml of the sample} = \frac{\text{Number of colonies} \times \text{Dilution factor}}{\text{Volume of the sample plated}}$$

$$\text{Number of colonies/ml of the sample} = \frac{150 \times 10^4}{1}$$

$$= 150 \times 10^4 \text{CFU/ml}$$

### 5.1.7.Identification of heterotrophic bacteria isolated from the gut of *Eisenia foetida*

The bacterial isolates were identified by various morphological and biochemical characterizations (Table 12) (Plate 4A, 4B, 4C). Among the two isolates obtained from the nutrient agar media, all isolates were non motile, gram negative rods with methyl red positive, acid slant acid butt in TSI test, catalase positive and citrate utilization positive.

### 5.1.8.Identification of fungi isolated from the gut of *Eisenia foetida*

From the RBA plates fungal isolate were obtained. They were identified by microscopic examination by LCB mounting technique and by colony morphology (Table 13) (Table 7A). They showed blue green colony on RBA plates.

### 5.1.9.Identification of heterotrophic bacteria isolated from the gut of *Eudrilus eugineae*

The bacterial isolates were identified by various morphological and biochemical characterizations (Table 14) (Table 5A, 5B, 5C, 5D). Among the two isolates obtained from the nutrient agar media, all isolates were non motile, gram negative rod with indole positive methyl red positive, citrate and catalase positive , acid butt alkaline slant in TSI test.

### 5.1.10.Identification of fungi isolated from the gut of *Eudrilus eugineae*

From the RBA plates fungal isolate were obtained. They were identified by microscopic examination by LCB mounting technique and by colony morphology (Table 15) (Plate 7B). They showed grey colour colony on RBA plates.

### 5.1.11.Identification of heterotrophic bacteria isolated from the gut of *Perionyx excavatus*

The bacterial isolates were identified by various morphological and biochemical characterizations (Table 16)(Plate 6A, 6B, 6C, 6D). Among the two isolates obtained from the nutrient agar media, all isolates were non motile, gram positive cocci with methyl red positive, acid slant acid butt in TSI test, catalase positive and citrate utilization positive.

### 5.1.10.Identification of fungi isolated from the gut of *Perionyx excavatus*

From the RBA plates fungal isolate were obtained. They were identified by microscopic examination by LCB mounting technique and by colony morphology (Table 17)(Plate 7C). They showed black colour colony on RBA plates.

**Table 6. Enumeration of Heterotrophic Bacteria isolated from the gut of *Eisenia foetida***

| S.NO | Dilution | CFU/ml | | |
|---|---|---|---|---|
| | | Original | Duplicate | Average |
| 1. | $10^{-4}$ | 205 | 196 | 200.5 |
| 2. | $10^{-5}$ | 89 | 84 | 86.5 |
| 3. | $10^{-6}$ | 51 | 48 | 49.5 |

**Table 7. Enumeration of Fungi isolated from the gut of *Eisenia foetida***

| S.NO | Dilution | CFU/ml | | |
|---|---|---|---|---|
| | | Original | Duplicate | Average |
| 1. | $10^{-4}$ | 185 | 174 | 179.5 |
| 2. | $10^{-5}$ | 33 | 38 | 35.5 |
| 3. | $10^{-6}$ | 18 | 21 | 19.5 |

**Table 8. Enumeration of Heterotrophic Bacteria isolated from the gut of *Eudrilus eugineae***

| S.NO | Dilution | CFU/ml | | |
|---|---|---|---|---|
| | | Original | Duplicate | Average |
| 1. | $10^{-4}$ | 191 | 169 | 180 |
| 2. | $10^{-5}$ | 55 | 69 | 65 |
| 3. | $10^{-6}$ | 33 | 21 | 27 |

**Table 9. Enumeration of Fungi isolated from the gut of *Eudrilus eugineae***

| S.NO | Dilution | CFU/ml | | |
|---|---|---|---|---|
| | | Original | Duplicate | Average |
| 1. | $10^{-4}$ | 199 | 179 | 189 |
| 2. | $10^{-5}$ | 98 | 61 | 79.5 |
| 3. | $10^{-6}$ | 23 | 11 | 17 |

**Table 10. Enumeration of Heterotrophic Bacteria isolated from the gut of *Perionyx excavatus***

| S.NO | Dilution | CFU/ml | | |
|---|---|---|---|---|
| | | Original | Duplicate | Average |
| 1. | $10^{-4}$ | 256 | 239 | 247.5 |
| 2. | $10^{-5}$ | 222 | 201 | 211.5 |
| 3. | $10^{-6}$ | 156 | 130 | 143 |

**Table 11. Enumeration of Fungi from the gut of *Perionyx excavatus***

| S.NO | Dilution | CFU/ml | | |
|---|---|---|---|---|
| | | Original | Duplicate | Average |
| 1. | $10^{-4}$ | 156 | 144 | 150 |
| 2. | $10^{-5}$ | 55 | 43 | 49 |
| 3. | $10^{-6}$ | 24 | 32 | 28 |

**Table 12. Identification of heterotrophic bacteria isolated from the gut of *Eisenia foetida***

| S NO | Identification tests | $FB_1$ | $FB_2$ |
|---|---|---|---|
| 1. | Gram staining | Gram negative rods | Gram negative rods |
| 2. | Motility | Non motile | Non motile |
| 3. | Indole test | negative | negative |
| 4. | Methyl red test | positive | positive |
| 5. | Voges proskauer test | negative | negative |
| 6. | Citrate utilization test | positive | positive |
| 7. | Triple Sugar Iron agar test | Acid slant acid butt | Acid slant acid butt |
| | | | |

| | | | |
|---|---|---|---|
| 8. | Catalase test | positive | positive |
| 9. | Oxidase test | positive | positive |
| 10. | Starch hydrolysis test | negative | negative |

**Table 13. Identification of fungi isolated from the gut of *Eisenia foetida***

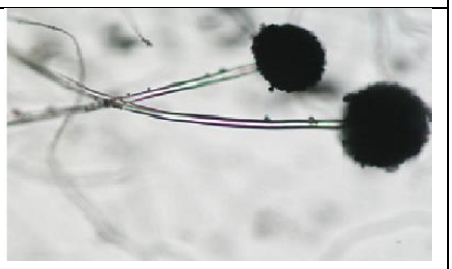
| S NO | Isolate | Colony morphology | Microscopic examination by LCB mounting technique |
|---|---|---|---|
| 1. | $FF_1$ | Grey colour colony |  |

**Table 14. Identification of heterotrophic bacteria isolated from thee gut of *Eudrilus eugineae***

| S NO | Identification tests | $EB_1$ | $EB_2$ | $EB_3$ |
|---|---|---|---|---|
| **Morphology characterization test** | | | | |
| 1. | Gram staining | Gram negative rods | Gram negative rods | Gram negative rods |
| 2. | Motility | Non motile | Non motile | Non motile |
| | | | | |

| Biochemical characterization | | | | |
|---|---|---|---|---|
| 3. | Indole test | positive | positive | positive |
| 4. | Methyl red test | positive | positive | positive |
| 5. | Voges proskauer test | negative | negative | negative |
| 6. | Citrate utilization test | positive | positive | positive |
| 7. | Triple Sugar Iron agar test | Acid butt alkaline slant | Acid butt alkaline slant | Acid butt alkaline slant |
| 8. | Catalase test | positive | positive | positive |
| 9. | Oxidase test | negative | negative | negative |
| 10. | Starch hydrolysis test | negative | negative | negative |

**Table 15. Identification of fungi isolated from the gut of *Eudrilus eugineae***

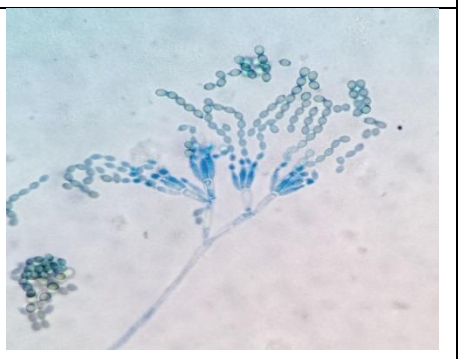
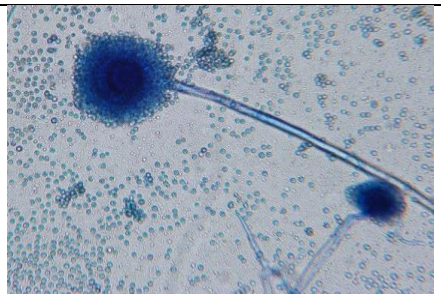
| S NO | Isolate | Colony morphology | Microscopic examination by LCB mounting technique |
|---|---|---|---|
| 1. | $EF_1$ | Blue green colony |  |

**Table 16. Identification of heterotrophic bacteria isolated from the gut of *Perionyx excavatus***

| S NO | Identification tests | $PB_1$ | $PB_2$ | $PB_3$ |
|---|---|---|---|---|
| **Morphology characterization test** | | | | |
| 1. | Gram staining | Gram +ve cocci | Gram +ve cocci | Gram +ve cocci |
| 2. | Motility | Non motile | Non motile | Non motile |
| **Biochemical characterization** | | | | |
| 3. | Indole test | negative | negative | negative |
| 4. | Methyl red test | positive | positive | positive |

| | | | | |
|---|---|---|---|---|
| 5. | Voges proskauer test | negative | negative | negative |
| 6. | Citrate utilization test | positive | positive | positive |
| 7. | Triple Sugar Iron agar test | Acid slant acid butt | Acid slant acid butt | Acid slant acid butt |
| 8. | Catalase test | positive | positive | positive |
| 9. | Oxidase test | negative | negative | negative |
| 10. | Starch hydrolysis test | negative | negative | negative |

**Table 17. Identification of fungi isolated from the gut of *Perionyx excavates***

| **S NO** | **Isolate** | **Colony morphology** | **Microscopic examination by LCB mounting technique** |
|---|---|---|---|
| 1. | $PF_1$ | Black colour colony |  |

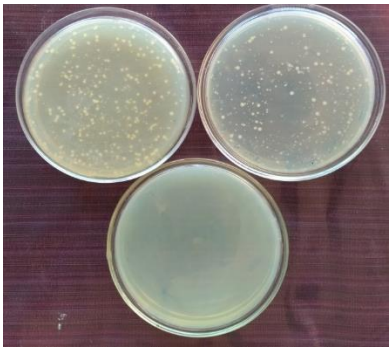


**Plate 1A: Enumeration of Heterotrophic Bacteria isolated from the gut of *Eisenia foetida* (dilution = $10^{-4}$)**

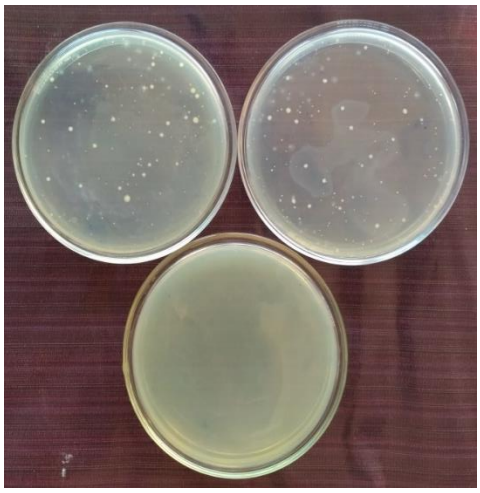


**Plate 1B: Enumeration of Heterotrophic Bacteria isolated from the gut of *Eisenia foetida* (dilution = $10^{-5}$)**

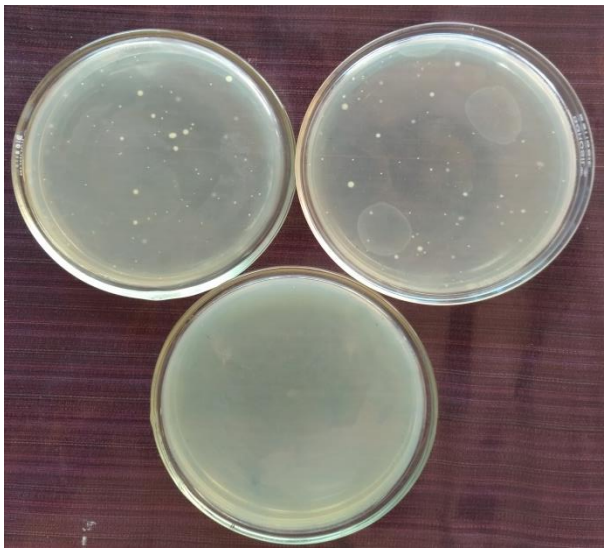


**Plate 1C: Enumeration of Heterotrophic Bacteria isolated from the gut of *Eisenia foetida* (dilution = $10^{-6}$)**

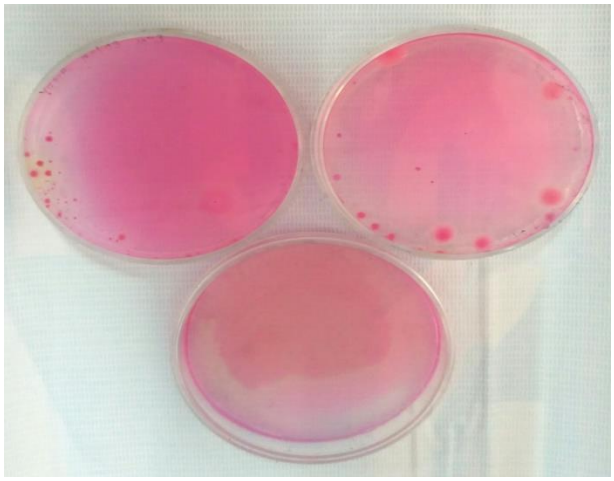


**Plate 1D: Enumeration of Fungi isolated from the gut of *Eisenia foetida* (dilution = $10^{-4}$)**

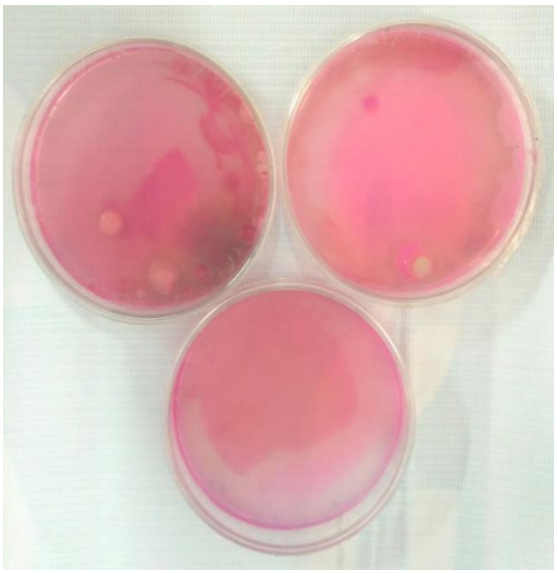


**Plate 1E: Enumeration of Fungi isolated from the gut of *Eisenia foetida* (dilution = $10^{-5}$)**

**Plate 1F: Enumeration of Fungi isolated from the gut of *Eisenia foetida* (dilution = $10^{-6}$)**

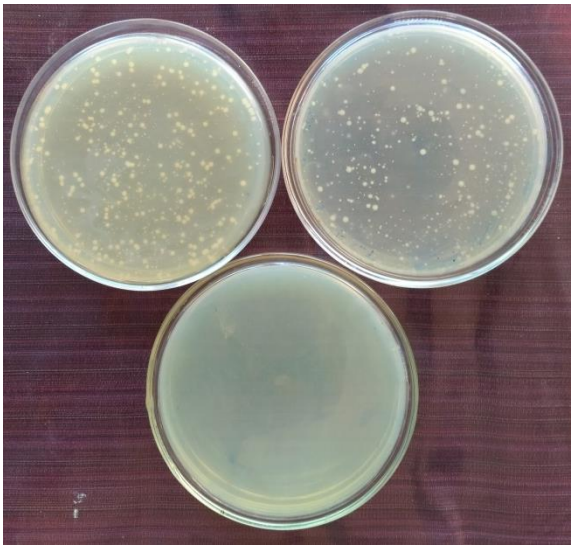


**Plate 2A: Enumeration of Heterotrophic Bacteria isolated from the gut of *Eudrilus eugineae* (dilution = $10^{-4}$)**

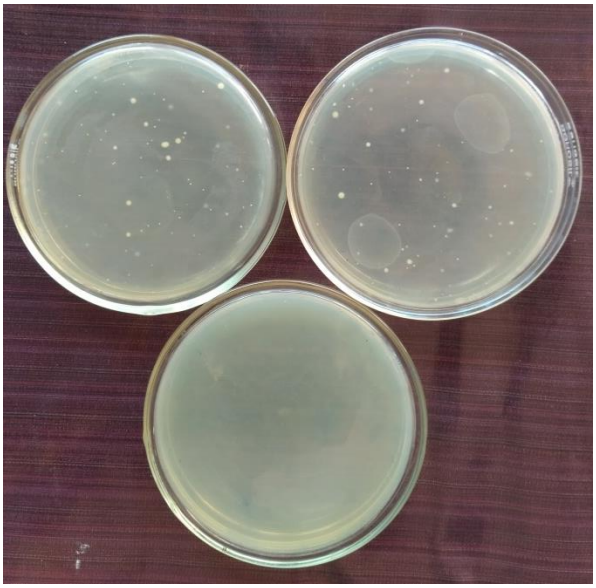


**Plate 2B: Enumeration of Heterotrophic Bacteria isolated from the gut of *Eudrilus eugineae* (dilution = $10^{-5}$)**

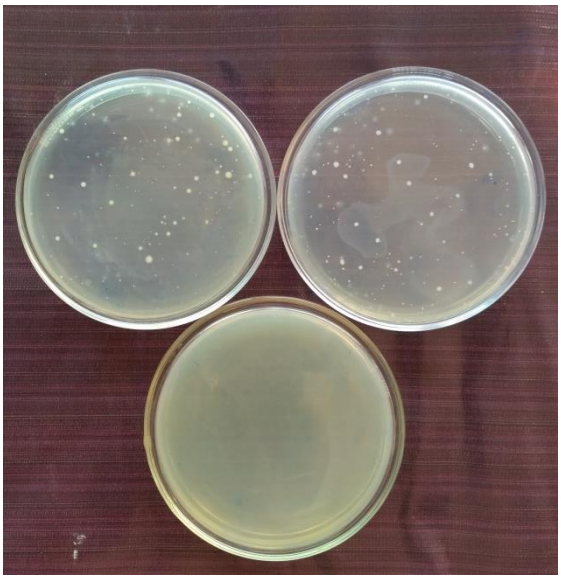


**Plate 2C: Enumeration of Heterotrophic Bacteria isolated from the gut of *Eudrilus eugineae* (dilution = $10^{-6}$)**

**Plate 2D: Enumeration of Fungi isolated from the gut of *Eudrilus eugineae* (dilution = $10^{-4}$)**

**Plate 2E: Enumeration of Fungi isolated from the gut of *Eudrilus eugineae* (dilution = $10^{-5}$)**

**Plate 2F: Enumeration of Fungi isolated from the gut of *Eudrilus eugineae* (dilution = $10^{-6}$)**

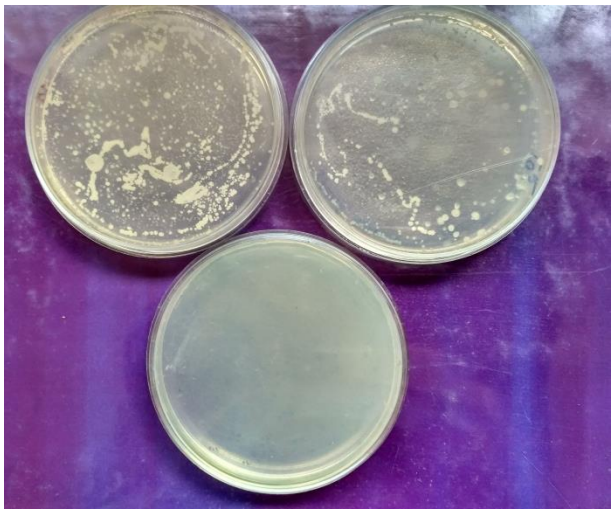


**Plate 3A: Enumeration of Heterotrophic Bacteria isolated from the gut of *Perionyx excavatus*(dilution = $10^{-4}$)**

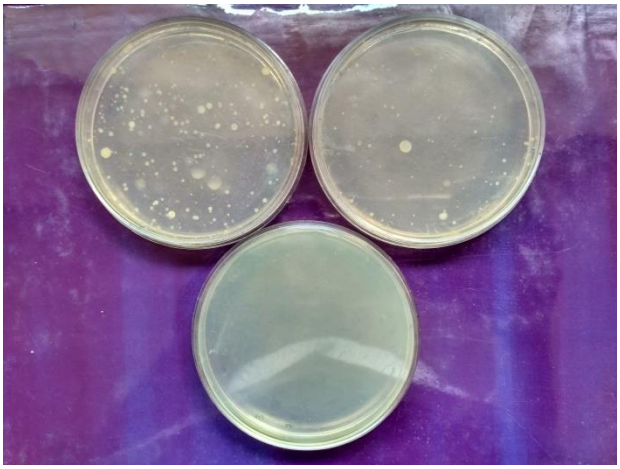


**Plate 3B: Enumeration of Heterotrophic Bacteria isolated from the gut of *Perionyx excavatus*(dilution = $10^{-5}$)**

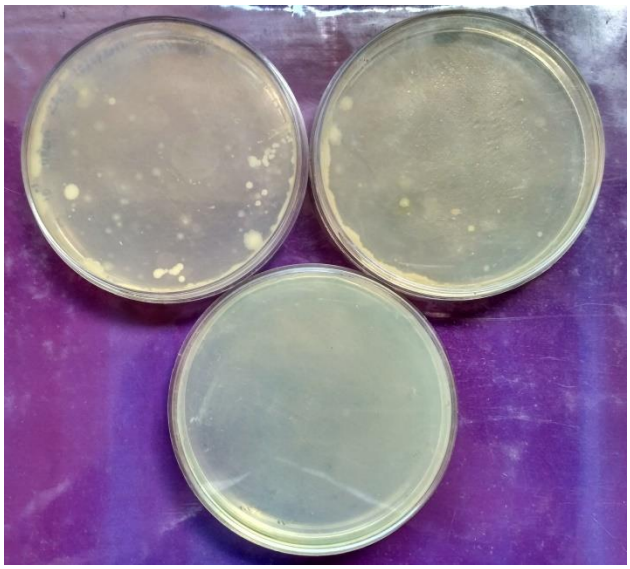


**Plate 3C: Enumeration of Heterotrophic Bacteria isolated from the gut of *Perionyx excavatus*(dilution = $10^{-6}$)**

**Plate 3D: Enumeration of Fungi isolated from the gut of *Perionyx excavatus*(dilution = $10^{-4}$)**

**Plate 3E: Enumeration of Fungi isolated from the gut of *Perionyx excavatus*(dilution = $10^{-5}$)**

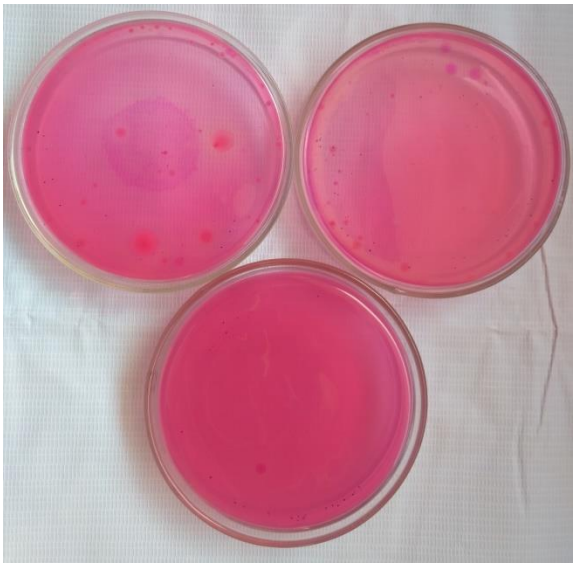


**Plate 3F: Enumeration of Fungi isolated from the gut of *Perionyx excavatus*(dilution = $10^{-6}$)**

## Identification of heterotrophic bacteria isolated from the gut of *Eisenia foetida*

### Biochemical tests

**Plate 4A: methyl red positive**

**Plate 4B: citrate utilization positive**

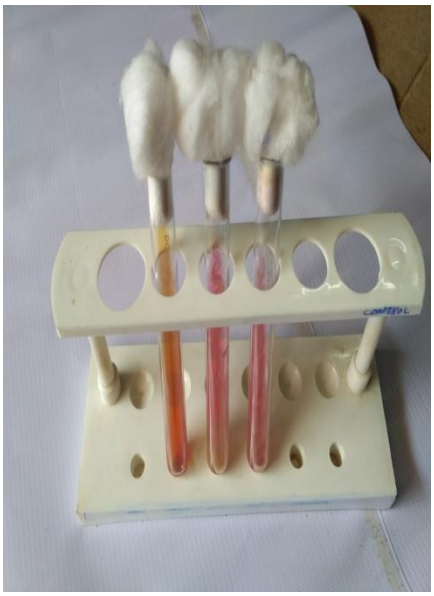


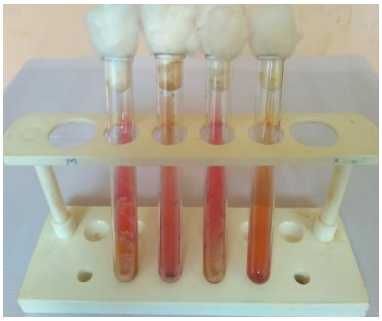
**Plate 4C: Acid slant acid butt,TSI test**

**Identification of heterotrophic bacteria isolated from the gut of *Eudrilus eugineae***

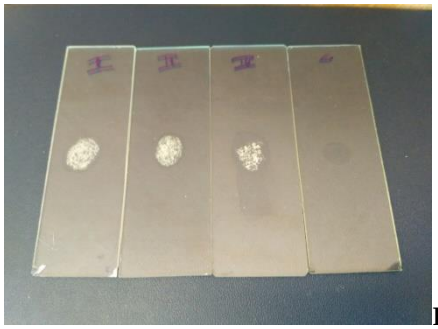
Biochemical tests

**Plate 5A: methyl red positive**

**Plate 5B: citrate utilization positive**



**5C:acid butt alkaline slant,TSI test**



**Plate**

**Plate 5D: catalase test positive**

**Identification of heterotrophic bacteria isolated from the gut of *Perionyx excavatus***

Biochemical tests

**Plate 6A: methyl red positive**

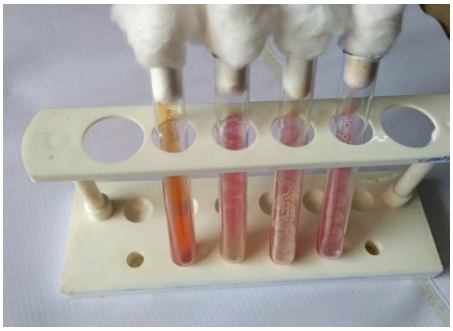


**Plate 6B: Acid butt acid slant, TSI test**

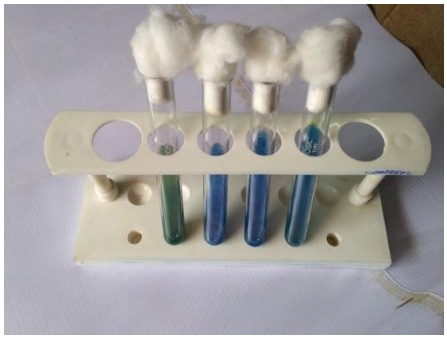


**Plate 6C: citrate utilization positive**

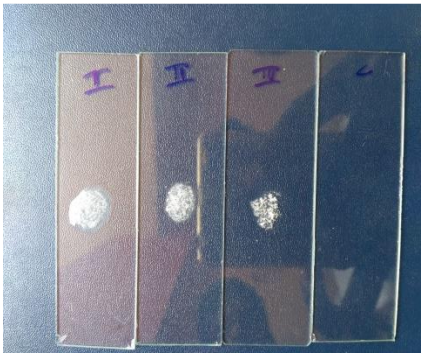



**Plate 6D: catalase positive**

**Identification of fungi isolated from the guts of *Eisenia foetida*, *Eudrilus eugineae*, *Perionyx excavatus***

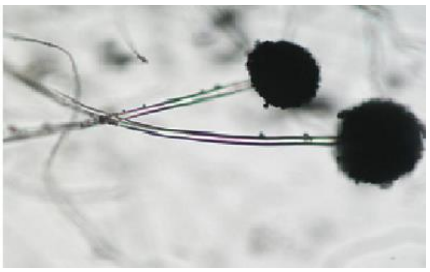


**Plate 7A: $FF_1$**

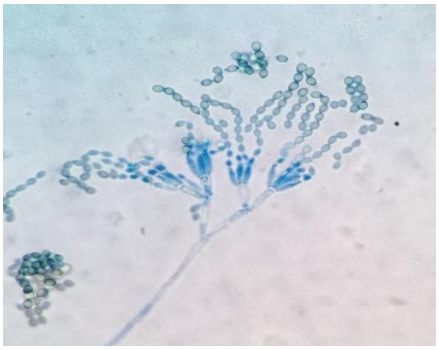


**Plate 7b: $EF_1$**

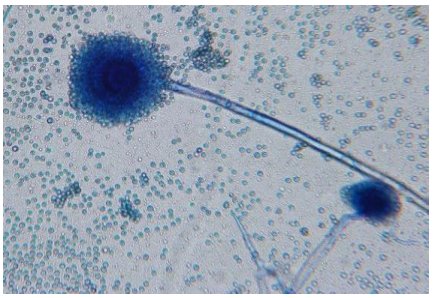


**Plate 7C: $PF_1$**

## 6. DISCUSSION

In the present study, the microbial analysis of the gut of three different earthworms were identified. The three different earthworms were *Eisenia foetida*, *Eudrilus eugineae* and *Perionyx excavates*. The worms gut was dissected and screened for the microbial analysis.The vermicomposting ability of the worm is enhanced by the gut microbes namely Heterotrophic bacteria, fungi and actinomycetes.

Earthworm possesses an immense bacterial diversity within their digestive/e tracts and is very little explored mainly because of the non-cultivatable character of a large quantity of microorganisms which mainly come from soil. Earthworm activity engineers the soil by forming extensive burrows which loosen the soil and makes it porous. ). Earthworm feeding reduces the survival of plant pathogens such as *Fusarium* sp. *and Verticillium dahliae* and increases the densities of antagonistic fluorescent pseudomonads and filamentous actinomycetes while population density of *Bacilli* and *Trichoderma* spp. remains unaltered (Elmer 2009). The gut microbes of earthworm were found to be responsible for the cellulase and mannose activities (Munnoli *et al*., 2010).

In the present study Heterotropic bacteria namely *Klebsiella* sp were identified in the gut of *Eisenia foetida* by Morphological and biochemical characterizations. Among the two isolates all showed same biochemical results and was found to be *Klebiella* sp. The bacteria namely *Pseudomonas* sp, *Enterobacter* sp, *Bacillus* sp, *Klebsiella* sp, were found significantly in the gut of earthworm *Eisenia foetida* (Uma maheswari and sudha, 2013). In the present study fungal isolate obtained from the gut of Eisenia foetida was identified by morphological (Colony morphology, LCB) examination. It was identified as *Mucor* sp. Parthasarathi *et al.* (2007) reported that the gut of *Eisenia foetida* has variety of fungal flora such namely *Aspergilulus* sp, *Mucor* sp, *Rhizopus* sp.

In the present study Heterotropic bacteria namely *Proteus* sp were identified in the gut of *Eudrilus eugineae* by Morphological and biochemical characterizations. Among the two isolates all showed same biochemical results and was found to be *Proteus* sp. The fungal isolate obtained from the gut of *Eudrilus eugineae* was identified by morphological (Colony morphology, LCB) examination. It was identified as *Penicillium* sp. Parthasarathi *et al*, (2007) identified the presence of *proteus*

spp in cowdung , but absent in the gut of *E. eugineae* reared on them. It could therefore be inferred that *Proteus* spp. was selectively injested by these earthworms as supplement of the lingocellulolitic digestion of wood from sawdust.

In the present study Heterotropic bacteria namely *Staphylococcus* sp were identified in the gut of *Perionyx excavates* by Morphological and biochemical characterizations. Among the two isolates all showed same biochemical results and was found to be *Staphylococcus* sp. Among the two isolates all showed same biochemical results and was found to be *Proteus* sp. The fungal isolate obtained from the gut of *Perionyx excavates* was identified by morphological (Colony morphology, LCB) examination. It was identified as Aspergilus niger. Samanta TT and Das A, (2016) isolated bacterial colonies from the gut of *Perionyx excavates* and identified them as *Bacillus* sp, *Staphylococcus* sp, *Enterococci*, *Micrococcus* sp and *Citrobacter* sp. Among the fungal isolates *Aspergillus* sp., and *P. boydii* were identified.

Thus the microorganisms present in the gut of earthworms are the major cause for casting. These organisms produce enzymes which are responsible for the breakdown of the waste products into valuable casting or vermicompost.

## 7.SUMMARY

In the present study the earthworms (*Eisenia foetida, Eudrilus eugineae, Perionyx excavatus*) were collected for the microbial analysis of their gut microflora.

The worm's harvested and dissected for the gut of the worm and the gut was homogenized in 0.85% NaCl (saline).

The microbial population in the gut was enumerated and found that bacteria stands in the first place and second place goes to fungus in all three species of earthworms.

The Heterotrophic bacteria in the gut was identified by morphological, biochemical characterization. The predominant bacteria in all three earthworms was identified and found that they were *Klebsiella* sp in *Eisenia foetida; Proteus* sp in *Eudrilus eugineae* and *Staphylococcus* sp in *Perionyx excavates*.

The fungal population in the gut of all three earthworms was identified and found that they were *Mucor* sp in *Eisenia foetida*; *Penicillium* sp in in *Eudrilus eugineae* and *Aspergilus niger* in *Perionyx excavates*.

Thus the earthworm composting ability is enhanced by the microbial activities in the gut. The earthworms can be used in the management of number of organic waste generated in agriculture, horticulture, rural industries including household section creating environmental population and problem.

Vermicomposting seems to be a natural tool for waste management since it convert the waste into wealth in form of compost and the environmental population will be mitigated. Thus the way of management of organic waste seems to be vermicomposting technique. Vermicomposting is an efficient eco-friendly method of waste management.

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# APPENDIX

**Media**

**Nutrient agar**

| | |
|---|---|
| Beef extract | 3g |
| Yeast extract | 3g |
| Peptone | 5g |
| Agar | 20g |
| Distilled water | 1000ml |
| pH | 7.5± 0.2 |

**Rosebengal agar**

| | |
|---|---|
| Dextrose | 10g |
| Peptone | 5g |
| Potassium phosphate | 1g |
| Magnesium sulphate | 5g |
| Rose bengal | 0.5g |
| Agar | 15g |
| Distilled water | 1000g |
| pH | 7.0±7. |

**Starch agar**

| | |
|---|---|
| Potato starch | 10g |
| Pancreatic digest of gelatin | 5.0g |
| Beef extract | 3.0g |
| Agar | 15g |
| Distilled water | 1000ml |
| pH | 6.2±0.2 |

**Simmons citrate agar**

| | |
|---|---|
| Ammonium di hydrogen phosphate | 1g |
| Di potassium phosphate | 1g |
| Sodium chloride | 20g |
| Sodium citrate | 2g |
| Magnesium sulphate | 0.2g |
| Agar | 15g |
| Bromothymol blue | 0.08g |
| Distilled water | 1000g |
| pH | 6.9±0.2 |

**Triple sugar iron agar**

| | |
|---|---|
| Beef extract | 3.0 |
| Yeast extract | 3.0g |
| Peptone | 15g |
| Protease peptone | 5g |
| Lactose | 10g |
| Sucrose | 10g |
| Dextrose | 1g |
| Ferrous sulphate | 0.2g |
| Sodium chloride | 200g |
| Sodium thiosulphate | 0.3g |
| Phenol red | 0.02g |
| Agar | 12g |
| Distilled water | 1000g |
| pH | 7.5±0.2 |

**Peptone broth**

| | |
|---|---|
| Peptone | 15g |
| Distilled water | 1000g |

| | |
|---|---|
| pH | 7.0±0.2 |
| **MR-VP broth** | |
| Peptone | 7g |
| Dextrose | 5g |
| Potassium phosphate | 5g |
| Sodium chloride | 200g |
| Distilled water | 1000ml |
| pH | 6.9±0.2 |

**Reagents**

| **Gram staining** | **g/ml** |
|---|---|
| **Crystal violet solution** | |
| Crystal violet | 2.0 |
| Ethanol | 20.0 |
| **Grams decolouriser** | |
| Ethanol 95% | 20.0 |
| **Gram's iodine** | |
| Iodine | 1.0 |

| | |
|---|---|
| Potasssium iodine | 2.0 |
| Distilled water | 300.0 |

**Safranine**

| | |
|---|---|
| Safranine | 0.2 |
| Ethanol | 10 |
| Distilled water | 90.0 |

**Methyl red Reagent**

| | |
|---|---|
| Methyl red | 1.0 |
| Ethanol | 300 |
| Distilled water | 200 |

**Voges Proskauer reagent**

**Barrit's A reagent**

| | |
|---|---|
| Alpha naphthol | 5.0 |
| Ethanol | 95.0 |

**Barrit's B reagent**

| | |
|---|---|
| KOH | 40.0 |
| Creatine | 0.3 |

**Kovac's reagent**

| | |
|---|---|
| P-di methylaminobenzaldehyde | 5.0 |
| Isoamyl alcohol | 75.0 |
| Conc.HCl | 25.0 |