

RESEARCH ARTICLE

ISOLATION AND CHARACTERIZATION OF GUT BACTERIAL SPECIES OF EARTHWORM *LAMPITO MAURITII*

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ABSTRACT

Lampito mauriti is an Anecic earthworm living in the topsoil and it is geophytophagous in nature. This earthworm is an important soil macro fauna as it has the dual role of an 'ecosystem engineer' due to the ability to build burrows as well as 'keystone species' in soil food webs because of its function in degradation of organic wastes. The present study investigates the gut of this earthworm to find the most predominant bacterium harbored inside. Gut contents were extracted and streaked on bacteriological media. The predominant type of colony was identified based on size, pigmentation, form, margin and elevation. Then different colonies were isolated and streaked separately to get pure colonies. The bacteria was subjected to several biochemical tests like Motility test, Gram staining, Methyl Red test, Voges-Proskauer test, Indole production test, Carbohydrate Fermentation test, Catalase test and Starch Hydrolysis test in order to identify the species. The bacterial species identification was done using Bergey's manual. The bacterial isolates such as *Micrococcus spp*, *Veillonella spp*, *Bacillus spp* and *Streptococcus spp* were identified.

Keywords: Anecic, *Lampito mauritii*, gut, microbes.

1. INTRODUCTION

Earthworm (phylum-annelida, class-oligochaeta) and comprise approximately 800 genera and 8000 species that account for upto 90% of invertebrate biomass present in soil (1). They are "key stone species" in soil food chain and "Ecosystem engineers" in maintaining the soil structure (2,3). Earthworms influence soil functions and processes, such as soil structure formation, soil carbon dynamics and bio geo chemical cycle. The Earthworm's gut plays a vital role in enriching the soil fertility. It is a tubular structure extending from mouth to the anus .It's different regions are the muscular pharynx, oesophagus, intestine and associative digestive glands. The analysis of gut contents in earthworms revealed the occurrence of different kinds of symbionts like micro fungi, bacteria, protozoa etc. (4). The gut bacteria enriches in total carbon, organic carbon and total nitrogen ratio of 24:1. Gut of Earthworms may be considered as ideal habitats for bacteria, because earthworm can directly regulate microbial population of their gut by consuming large amount of the soil. The micro organisms available in the gut of earthworm species are mostly related to the soil micro flora.

It is well established that the earthworm gut provides suitable condition for the development of bacterial colonies since earthworm castings contain significantly higher counts of bacteria than in surrounding soil (5). According to Atlavingte and

Lugankas (1971) earthworms increased the number of microbes in soil as much as five times. A number of researches have observed increased proliferation of a variety of micro organisms in the gut of earthworms, fungi in *P. millardi*, *L. mauritii* and *E. euginiae*, bacteria in *A.caliginosa*, *L.terrestris*, *L. mauritii* and actinomycetes in *L. terrestris*, *A. longa* and *A.caliginosa* and *L. rubellus*. Among the bacteria, vibrio species were shown to be selectively proliferated. But recent studies of parthasarathi have shown the selective proliferation to vary with type of food materials and species of earthworms. They have shown that there was a significant multiplication of micro organisms in the gut of *L. mauritii*, *E.euginiae* and *P.excavatus* reared on different substrates fungi (53-82% in clay loam soil), (15-56% in cow dung) and (11-54% in press mud), bacteria (29-59% in CLS), (62-75 in CD) and (50-68% in PM). Increase in microbial population might be due to the environmental conditions prevailing and nutritional status in the gut of earthworm as reported by Senapathi and Dash (6).

Similar to the occurrence of greater number of microbes in the gut of earthworms than the surrounding soil. The cast also contains more micro organisms particularly more number of fungi, actinomycetes, bacteria and higher enzyme activity were reported in the cast [7]. A significant increase of microbial population in the casts of different species of earthworms was reported by Parthasarathi et. al. (8). Especially earthworms have

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shown to enhance the spread of VAM (Vesicular Arbuscular Mycorrhizae), but also they support the survival of spores for a longer time. Earthworms were also found to help in the dispersal of nitrogen fixing bacteria, actinomycetes like *Frankia* and the rhizobium bacteria-*Bradyrhizobium japonium*. Since the vermicast and the vermicompost are good store houses of microbes their application to soil help greatly in the dispersal of microbes.

The total microbial population has enhanced more than 397% of microbial activity reflected by the enhanced activity of dehydrogenase. Among the microbes, bacteria have abundantly multiplied (386%) and actinomycetes though low in population, have also multiplied (151%) more or less equally with fungi (169%).

2. MATERIALS AND METHODS

2.1. Study area and sampling location

Coimbatore is the second largest city in the Indian state of Tamil Nadu. It is situated in the western corner of Tamil Nadu, a short distance from the border with the neighbouring state of Kerala, and is surrounded by the Western Ghats on all sides with reserve forests and the Nilgiri Biosphere reserve on the northern side.

The samples were collected from an agricultural field situated in Kanuvai, Nanjappapuram, Coimbatore.

2.2. Collection, Identification and Preservation

The earthworms were collected with the help of a rod and shovel by digging and were sorted out using hands. They were identified by Dr. P. Kathireswari. The collection was done in the month of September 2018 around 2 p.m. The soil was dug up to a depth of 30 – 70 cm and the earthworms were hand collected precautiously without any damage. The collected worms were washed in clean water and kept in a beaker with cellulose fiber in order to remove the soil and waste particles from its gut and one worm was preserved in 95% ethanol to observe the morphological characters.

2.3. Bacterial Colony Characterization

Colonies with different morphological appearances were observed and counted. The colony count technique had been routinely used, as it is easy to perform and can be adopted for the measurement of populations of any magnitude. The monographic works of Dash (9), Edwards (10), Ranganathan (11) were used for the identification of the worms and its morphological characters.

2.4. Isolation of bacteria from the earthworm gut

Each specimen to be dissected was washed with sterile distilled water and surface sterilized using 95% ethanol. The specimen was placed on the wax dissecting tray and was fixed longitudinally using sterile dissecting pins, one mounting the mouth and other the anus. The fine edge of a flamed pair of dissecting scissors was inserted into the ventral surface at the mouth region and the incision was made longitudinally along the earthworm till the anus. Sterile dissecting pins were used to hold down the earthworm on the board, stretching out the body wall to expose the internal structures.



Table:1 Bacterial colony characterization.

CHARACTERS	TYPES
Size	From extremely small (pin point) to large colonies (5-10 mm in diameter)
Margin or edge	Evenly circular, irregular, undulate, serrate, filamentous etc.
Surface texture	Smooth (shiny, glistening), Rough (dull, granular), Mucoid (slimy or gummy)
Elevation	Thin or thick, flat, raised, convex, Umbonate etc.
Optical feature	Opaque, translucent or opalescent.
Pigmentation	Yellow, pink, golden, white etc.

2.5. Serial dilution

0.95 grams of the earthworm's gut tissue is homogenised in 9 ml of distilled water using Mortar and Pistle, and 1 ml was transferred into a test tube

containing 9 ml of sterile distilled water. It is labelled as 10^{-1} . Like this 9 ml of distilled water was filled in 5 other test tubes and labelled as 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} . Then 1 ml of sample is taken from 10^{-1} using micro pipette and added into 10^{-2} test tube. From that 1 ml is taken and added into 10^{-3} test tube and serially diluted up to 10^{-6} test tube.

2.6. Spread plate method

1.5 grams of agar and 1.3 grams of nutrient broth was mixed and dissolved in 100 ml of distilled water. This nutrient agar medium was autoclaved at 121°C at 15 lbs. pressure for 15 minutes. The medium is transferred into the petri plates under aseptic condition in the laminar air flow chamber.

Then the petri plate upper lid is removed and about 0.1 ml of diluted sample from 10^{-1} and 10^{-2} dilutions is placed at the centre of the plate. The L rod is sterilized by dipping it in ethanol and heating it in flame. The glass rod is cooled and inoculum placed in the petri plate is spread uniformly by swirling the rod over the petri plate. The petri plates are inverted and incubated at 37°C for 24 hours.

2.7. Streak plate method

The nutrient agar medium was prepared and autoclaved at 121°C at 15 lbs. pressure for 15 minutes. The medium is poured into the petri plates under aseptic condition in the laminar air flow chamber. The sample is taken from the nutrient broth and placed on the medium near the rim of the plate.

The inoculation loop is sterilized, flamed and cooled and spread from one area to other area with parallel streaks, and care should be taken that streaks should not overlap each other. After looping, the plates were inverted and incubated at 37°C for 24 hours.

2.8. Gram staining:

Wipe the clean glass slide with 95% ethanol. Dry the slide and label it regarding which bacteria is stained. From the bacterial suspension broth take sterile loop full of broth culture on the slide. Spread by means of circular motion of the inoculating loop to about 1 cm in diameter.

Then place sterile water or saline solution on the slide using a dropper and gently stir on the slide. Allow the smear to air dry or heat fix it by passing the entire slide through the flame two to three times. Place the slide with heat fixed smear on the staining tray. Then gently flood smear with crystal violet reagent and let stand for 1 minute. Here crystal violet acts as the primary stain. Rinse the slide with

sterilized water. After this gently flood the slide with Grams Iodine solution and let stand for 1 minute. Grams Iodine acts as the mordant. Rinse the slide with sterile water.

Then decolourize the slide with 95% ethanol. Immediately rinse it with sterile distilled water. After this gently flood the smear with safranin reagent and let stand for 45 sec to 1 minute. Here safranin acts as the secondary stain. Rinse with sterile water. Dry the slide. Then view the slide under Oil Immersion Microscope. If the bacteria's were stained blue or violet then they are gram positive and if they are stained pink they are termed as gram negative.

2.9. Motility test:

Clean cavity slide was taken and placed on the table with depletion upper side. A cover slip was taken and wax was applied on four corners. A loopful of culture was transferred exactly at the centre of the cover slip. Cavity slide was placed on the cover slip and pressed gently. The preparation was lifted gently so that the culture drop is suspended in the form of hanging drop. The slide was observed under the light microscope at 45X magnification. The cell which is movable is called as motile.

2.10. Catalase test:

Nutrient agar medium was prepared. The medium was poured into culture tubes. It was sterilized by autoclaving at 15 lbs. pressure for 15 minutes. The nutrient agar slants were inoculated with bacterial suspension culture. The cultures were incubated at 37°C for 24 hours and 3 – 4 drops of hydrogen peroxide was added on the growth of each slant culture. The culture was observed for the appearance or absence of gas bubbles.

2.11. Indole production test

1% Peptone broth was prepared and sterilized using autoclave at 15 lbs. pressure for 15 minutes. The peptone broth was inoculated with bacterial suspension culture. The tubes were incubated at 37°C for 24 hours. After this 1 ml of Kovac's reagent was added. The tubes were shaken gently after intervals of 10 to 15 minutes. The tubes were allowed to stand for few minutes to permit the reagent to come to the top. The tubes were observed for cherry red layers in the top layer. The result is positive if the cherry layers are there and negative if not.

2.12. Methyl Red test

Methyl red broth was prepared and sterilized using autoclave at 15 lbs. pressure for 15 minutes. 5 ml of broth was poured in each tube. The tubes were inoculated with the bacterial suspension culture. All the tubes were incubated at 37°C for 24 hours. 5 drops of methyl red indicator was added to the tubes of each set. The change in colour of methyl red was observed. The result is positive if red colour appears and negative if yellow colour appears.

2.13. Voges-Proskauer test

Voges-proskauer broth was prepared and sterilized using autoclave at 15 lbs. pressure for 15 minutes. 5 ml of broth was poured into each tube. The tubes were inoculated with the bacterial suspension culture. All the tubes were incubated at 37°C for 24 hours. 12 drops of VP reagent-I and 2-3 drops of VP reagent-II was added to the tubes. The tubes were gently shaken for 30 seconds with the caps off to expose the media to oxygen. The tubes were kept aside for 15-30 minutes and observed for change in colour for the VP test. The result is positive if red colour appears and negative if yellow colour appear.

2.14. Carbohydrate Fermentation test

Fermentation medium was prepared with specific carbohydrate such as Glucose was used. The media was sterilized using autoclave at 15 lbs. pressure for 15 minutes. The medium was inoculated with bacterial suspension culture. The tubes were incubated at 37°C for 24 hours. The tubes were observed for the change in colour (i.e.) yellow (due to production of acid) or change in colour (i.e.) red and appearance of bubbles (dueto production of acid and gas).

2.15. Starch hydrolysis test:

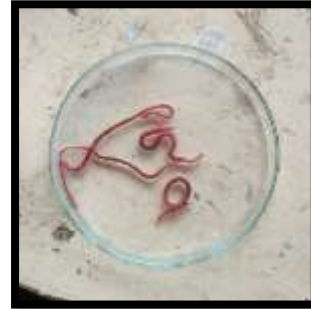
Starch agar medium was prepared and sterilized using autoclave at 15 lbs. pressure for 15 minutes. The media was poured into petri plates and allowed to solidify. The bacterial suspension culture was inoculated on to the plate with a sterile transfer loop. The plate was incubated at 37°C for 24 hours.

After incubation the plate was flooded with Gram's Iodine. Plate was observed for clear zone around the inoculated organism. The result is positive if there is a clear zone around the line of growth and negative if there is dark blue colouration of the medium.

3. RESULTS

In the present study of earthworms made in Kanuvai, Coimbatore, Tamil Nadu reveals that the

earthworm species is *Lampito mauritii* classified under the phylum Annelida, Class Clitellata, Sub class oligochaeta and order Megascolecidae and has been identified under morphological and taxonomical characterization.



Colony characterization of five bacterial isolates

Bacterial isolate 1–Large in size, light yellow in colour, irregular form, undulate margin, raised elevation and translucent.

Bacterial isolate 2– Moderate in size, white in colour, circular form, circular margin, convex elevation and opaque.

Bacterial Isolate 3 – Small in size, white in colour, circular form, serrate margin, raised elevation and translucent.

Bacterial Isolate 4 – Small in size, white in colour, irregular form, undulate margin, umbonate elevation and translucent.

Bacterial Isolate 5 – Moderate in size, white in colour, irregular form, filamentous margin, raised elevation and opaque.

Table: 2 Colony forming units of five bacterial isolates

Bacterial isolates	Colony Forming Units (approx.)
Isolate 1	10
Isolate 2	45
Isolate 3	54
Isolate 4	89
Isolate 5	30

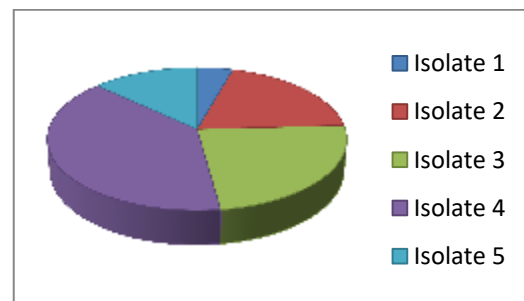


Fig. 1 Colony forming units

The bacterial isolates consisted of variety of gram negative cocci and gram positive rods and cocci. The five isolated organisms was identified and confirmed as *Micrococcus spp*, *Streptococcus spp*, *bacillus spp*

and *veillonella spp* by using above biochemical tests (Table 2). All these bacterial species were identified using Bergey's manual.

Table 3. Bio chemical features of Bacterial isolates 1 – 5

Morphology and biochemical test	Isolates				
	1	2	3	4	5
Gram staining	Gram positive cocci	Gram positive cocci	Gram positive cocci	Gram positive rod	Gram negative cocci
Motility	Non motile	Non motile	Non motile	Motile	Non motile
Catalase	+ve	-ve	+ve	+ve	-ve
Indole	-ve	+ve	+ve	-ve	-ve
Methyl Red	-ve	+ve	+ve	-ve	-ve
Glucose fermentation	-ve	+ve	+ve	+ve	+ve
Voges-Proskauer	-ve	-ve	-ve	-ve	-ve
Starch Hydrolysis	+ve	-ve	+ve	+ve	-ve

During gut passage, the *in situ* factors of the earthworm gut, which include anoxia and high concentration of organic substances, appear to greatly stimulate a subset of ingested soil microbes (4). In the present study the bacterial colony having irregular form, small in size, white in colour, undulate margin, Umbonate elevation and translucent in nature was the predominant one in all of the five isolates. The bacterial isolate include *Bacillus spp*. It is assumed to play important roles in decomposition of organic matter and thereby soil quality enrichment.

4. DISCUSSION

The five isolated organisms was identified and confirmed as *Micrococcus spp*, *Streptococcus spp*, *bacillus spp* and *veillonella spp* by using biochemical tests. All these bacterial species were identified using Bergey's manual. In the present study it was found that bacterial species *Bacillus subtilis* was the predominant bacteria found the in the gut of *Lampito mauritii*.

But the predation of bacteria in *Lampito mauritii* done by Parthasarathi et al. (8) found different species of bacteria's like *Pseudomonas aeruginosa*, *Bacterium antitratum*, *Mima polymorpha*,

Enterobacter aerogenes, *Enterobacter cloacae*, *Proteus mirabilis*, *E. coli*, *Micrococci*, *Bacillus subtilis* and *Streptomyces spp*. Their study has conclusively established the dominant occurrence of *Klebsiella pneumoniae* and *Morganella morganii* in the gut of *Lampito mauritii*. The number of *Bacillus cereus* and *Bacillus idozus* were found to be in greater extent in the gut of the earthworm *Lampito mauritii*.

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