A Study of the Biodiversity of Gut Microbiota of Eisenia Fetida in Response to Contamination by Pesticides and Metal

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Abstract **- The use of chemical pesticides and fertilizers in Indian agriculture has increased in recent years. Also, human activities have resulted in an increase in the concentrations of metals, metalloids and pesticides in urban and rural soils. These lead to disruptions to the natural biogeochemical cycles of metals and pesticides, thus, resulting into toxic effects on the beneficial fauna and flora. The present study observed the effects of two commercial pesticides Pendimetahlin (LC50-0.16 mg/Kg) and Chlorpyrifos (LC50- 0.062 mg/Kg) and the metal zinc on the gut microflora of the earthworm *Eisenia fetida* (Tiger worm). The pesticides and metal were applied in two different doses. Firstly, the bacterial and fungal diversity from the gut of *Eisenia fetida* was isolated and identified depending upon the morphological and biochemical tests. The isolated strains were identified as *Bacillus* sp., *Clostridium* sp., *Vibrio* sp. and *Staphylococcus* sp., while the fungi isolates were identified as *Penicillium* sp., *Ulocladium* sp., *Exophiala* sp. and *Candida* sp. Out of these isolated microbes, all the fungal isolates were found to produce the enzyme cellulase, xylanase and laccase. It has been observed that the CFU of these fungal isolates were reduced significantly when the earthworms were subjected to the pesticides and metals. Both the pesticides and metals were proved to be more detrimental for the enzyme-producing fungal isolates than the bacterial ones. Hence, this study throws light on the adverse effects of the use of these chemical pesticides and metal contamination on the beneficial microbes within the earthworm's gut.**

Index Terms **- *Eisenia fetida*, gut microflora, pesticides, metal, enzymes.**

1.INTRODUCTION

Earthworms play an important part in soil construction and recycling of organic waste. They are a part of a network of organisms that turn waste into nutrient rich soil. These earthworms harbor a variety of microorganisms inside its gut which play an important role in the earthworm's activities. Among these microorganisms some bacteria, fungi and yeast are the primary decomposer of organic wastes [1]. Microbes are responsible for the biochemical degradation of the organic matter. Earthworms are the important drivers of this process, conducting the substrate (organic wastes), producing congenital conditions for the activities of microbes and altering biological activity [2]. The microorganisms and the earthworms act symbiotically to accelerate and enhance the decomposition of organic matter and as a consequence, humification and mineralization takes place, which results in the availability of nutrients for plants [3, 4, 5].

Earthworms ingest microorganisms present in the soil along with organic residues from the soil and the population of these microorganisms may increase during their passage through the worm's intestinal tract [6, 7]. Due to these characteristics, earthworms act as vectors for the dispersal of soil microorganisms [8]. Several differences exist between the gut condition of earthworms and the soil environment [9]. To survive throughout the gut passage these microorganisms must adapt to the anaerobic and physicochemical gut conditions [10], the lysis of microbes by digestive enzymes secreted by the earthworm, and the inhibition of bacteria by inhibitory

substances secreted by other bacteria [11]. Therefore, to facilitate the use of earthworms as vectors for the dispersal of beneficial microorganisms, and to increase the soil fertility, the intestinal microorganisms in earthworms must be characterized. Earthworms are found in soils containing high levels of metals [12, 13, 14] and these earthworms represent a major constituent of soil fauna. Earthworms, generally, increase the mobility and availability of metals and metalloids in the soil [15] and this may result in greater concentrations of metals leaching out of the soil into ground water [16]. They may also reduce the efficiency of soil remediation by mobilizing recalcitrant metals [17]. Metal toxicity in soils are determined by the bioavailability rather than total metal concentrations [18] and this depends on mobility and speciation in the living soil environment [19, 20, 21]. There are many studies that have reported the impact of metals in soil and also on the inhabiting earthworms and their gut-associated microflora [22].

The use of pesticides in agricultural practices often results in loss of biodiversity of gut microbiota of the earthworms[23]. The significance of earthworms has been well acknowledged but yet it requires sufficient data on the toxicity of specific pesticides to such non-target organisms in order to select chemicals that induce less harm to them. Most pesticides have a detrimental effect on several species of earthworms.

In this study, we chose the earthworm *Eisenia fetida* as the model organism, because this species significantly increases the decomposition of organic matter and rapidly increases its weight [24]. Due to these properties, attention has been given to the use of earthworm *Eisenia fetida* for the treatment of biosolid wastes [25]. The subsequent goal of the present study was to identify the microfloral population of the gut of *Eisenia fetida* as this species has been employed as a biological indicator of organic matter stabilization [26], and to observe the effect of two commonly used pesticides and metal zinc on them.

2.MATERIALS AND METHODS

2.1. Organism for the study

Eisenia fetida specimens were bought from Mecheda (Purba Medinipur).

2.2. Maintenance of the earthworm species

The worms were stored in plastic containers with pores, filled with suitable quantity of wet compost soil.

2.3. Test pesticides and doses

Commercially available pesticides were used in the present study. Technical information and doses regarding these pesticides has been given in table I.

2.4. Test metal and its doses

The metal we used for this experiment is Zinc (Zn). The pollutant was added in form of $ZnSO_4$. The earthworms were exposed to two doses of this metal i.e. Dose I- 60mg/Kg of the soil and Dose II- 120mg/Kg of the soil (table I).

Table I: Table showing the Exposure Dose and Time period for both the pesticides and metal for *Eisenia fetida*

Exposed to		Days of Exposure
Pesticide 1 Pendimethalin (LC50-0.16mg/Kg)		3 Days
Dose I-¼LC50	Dose II-½ LC50	
Pesticide 2 Chlorpyrifos (LC50-0.062mg/Kg)		3 Days
Dose I-¼LC50	Dose II-½ LC50	
Metal Zinc		7 Days
Dose I- 60mg/Kg	Dose II- 120mg/Kg	

2.5. Method of exposure to the metal and pesticides

Thirty clitellate adult *Eisenia fetida* worms weighing 120-200mg in three replicates exposure chamber, containing 1 Kg dry mass of artificial soil and 4 Kg of feed material were chosen for the experiment. The earthworms were then exposed separately to the respective chambers for a period of 7 days for metals and 3 days for the pesticides. A control set was also maintained without the metal and pesticides.

2.6. Preparation of the earthworm extract

Complete gut of each earthworm was dissected out, weighed and homogenized in sterile 0.85% NaCl solution. We maintained the replicates in microbial analysis process also by analysing the gut content of each specimen, present in each of the container, separately. The resultant homogenates were then used as the extract for the microfloral isolation.

2.7. Isolation of gut microflora

The resulting suspension of each earthworm from each test condition was serially diluted with sterile water and used as inoculums. About 0.1ml of the inoculums of each earthworm was separately inoculated into different agar media for growth of bacteria and fungi. The plates were then incubated at 30-37°C for 18-24 hours for bacteria, 25-28°C for 4-7 days for fungi, 30-37°C for 10-12 days for actinobacteria and 25-37°C for 12-14 days for yeast.

2.8. Enumeration of the isolated bacteria

The CFU was done for each isolated strain of bacteria by dilution pour plate method [27].

2.9. Identification of the microflora

To identify the bacteria, actinobacteria and yeast at genus level, Gram's staining, spore staining and various biochemical tests were performed as described in Mahon and Manuselis [28].

All the fungal isolates were identified using Lactophenol blue staining and morphological characterization (color and texture of fungal colony). The morphological and cultural features of each fungus were compared with descriptions given by Bryce [29] and Kwon-Chung and Bennett [30] for identification.

2.10. Preparation of hierarchical clustering of the bacterial isolates

The hierarchical cluster analysis of the isolated bacterial strains was done based on the phenotypic and biochemical characteristics of the isolates.

2.11. Effect of Zinc metal and the pesticides on isolated gut microflora

The CFU of the isolated microflora was calculated for the control and the two doses of metal and both the pesticides to see if the metal and pesticide exposure had any detrimental effect on them.

3.RESULTS

The results were observed after the respective days of incubation. Different types of bacteria, fungus and yeast were observed. Firstly, they were characterized in order to identify them as a part of earthworm's gut microbial population. A total of 9 bacteria, 12 fungi and 2 yeast were obtained, out of which only 5 bacteria (EB1, EB2, EB3, EB4, EB5), 4 fungi (EF1, EF2, EF3, EF4) and 1 yeast (EF5) were identified depending on their morphological and biochemical characteristics.

2.12. **Characterization of Bacteria:**

Out of the five identified bacteria, three were found Gram positive and two were found to be Gram negative (Fig. 1). All the isolates gave negative result for VP test except for EB3 which is comma-shaped bacterium. All the isolates were positive for catalase activity and starch hydrolysis property. All the isolates were found to ferment sucrose except isolate EB2. The results for biochemical tests for the bacterial isolates have been summarized below in Table II.

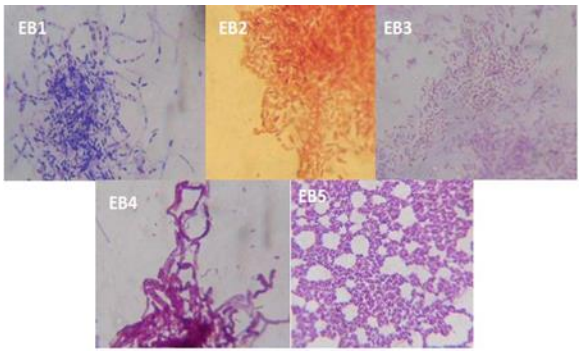

Fig 1: Gram staining of the isolated bacterial strains (Magnification 1000X)

Table II: Summarized results of biochemical tests performed on the obtained bacterial isolates

Biochemical tests	Bacterial Isolates				
	EB1	EB2	EB3	EB4	EB5
Gram's Staining	+	-	-	+	+
Shape	Bacillus	Bacillus	Comma	Bacillus	Cocci
Cultural Characteristics on agar plate	Off-white, very irregular extremities	Off-white, irregular raised surface	White, shiny, smooth edged	Abundant, opaque, cream colored, irregular surface	White, shiny
Catalase test	+	+	+	+	+
Oxidase test	+	-	+	+	+
Starch hydrolysis test	+	+	+	+	+
Sucrose fermentation test	+	-	+	+	+
Voges-Proskauer test	-	-	+	-	-

2.13. Characterization of Fungi, Actinobacteria and Yeast:

All the fungal isolates were cultured on Sabouraud's Dextrose agar and their morphology was found to be quite different from each other (Fig 2).

Lactophenol cotton blue staining was done in order to study their filament structure (Fig 3).

Depending on the above characteristics of fungi, the results have been summarized below in Table III.

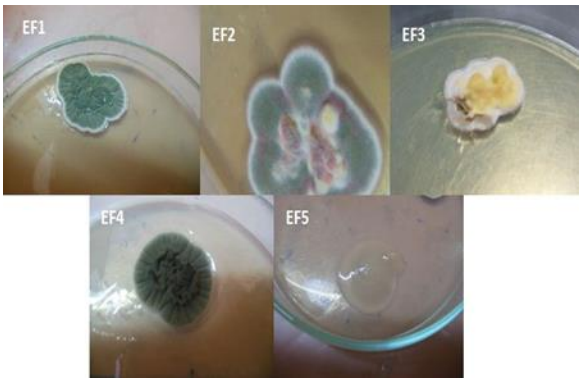

Fig 2: Pure cultures of the fungal isolates on SDA plates for morphological characterization

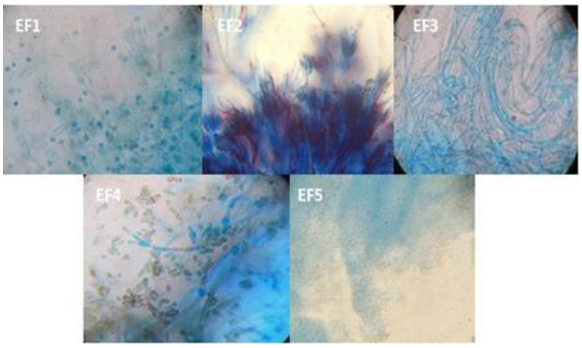

Fig 3: Lactophenol cotton blue staining of the fungal isolates (Magnification 1000X)

Table III: Table showing the results of biochemical and morphological study on the fungal isolates

Biochemical and morphological study	Fungal Isolates				
	EF1	EF2	EF3	EF4	EF5
Gram's staining	NA	NA	NA	NA	NA
Lactophenol cotton blue staining	Blue conidiophores observed	Branched conidiophores, Red blue in color	Highly Branched mycelia	Long spores, hyphae	Globose, elliptical and oval cells
Colony Color	Greenish with white border	Greenish, red-centered	White and	Grayish yellow	Shiny off-white
Fungal texture	Circular, rough	Circular, furry	Irregular edges, fluffy	Irregular to highly wrinkled colony	Shiny, slimy, irregular

2.14. CFU calculation-

CFU has been calculated for the isolated bacteria by dilution plating method. The CFU of the bacterial isolates EB1, EB2, EB3, EB4 and EB5 were observed as 32 X 10^6, 49X 10^6, 102 X 10^6, 17 X $10^6$and 154 X 10^6 respectively.

2.15. Probable Nomenclature based on the above studies-

Based on the observations made in the biochemical tests and on their comparisons with Mahon and Manuselis (1995) and Bryce and Kwon-Chung (1992), the probable nomenclature of the isolates has been tabulated in Table IV.

Table IV: Table showing the Probable nomenclature of the isolated microflora from the gut of *Eisenia fetida*

Isolates	Probable nomenclature
EB1	*Bacillus cereus*
EB2	*Clostridium sp.*
EB3	*Vibrio comma*
EB4	*Bacillus subtilis*
EB5	*Staphylococcus sp.*
EF1	*Penicillium notatum*
EF2	*Penicillium sp.*
EF3	*Ulocladium sp.*
EF4	*Exophiala werneckii*
EF5	*Candida albicans*

2.16. Cluster analysis of the isolated bacterial isolates based on the phenotypic and biochemical isolates-

A hierarchical clustering is performed using the similarity matrix with the help of the UPGMA (Unweighted Pair Group Method of Averages) and the result is visualized in a dendograms, which reveals the similarity among the strains. (Table V, Fig 4). Also the

similarity matrix has been calculated using Jaccard index (Table VI).

Table V: Table containing phenotypic and biochemical characters of each investigated strain

Characteristics	Strains				
	EB1	EB2	EB3	EB4	EB5
Cell shape*	0	0	0	0	1
Gram staining**	0	1	1	0	1
Catalase Test	0	0	0	0	0
Oxidase Test	0	1	0	0	0
Starch hydrolysis Test	0	0	0	0	0
Sucrose Fermentation Test	0	1	0	0	0
Voges-Proskauer Test	1	1	0	1	1

*coccus(1); rod(0)

**positive (0); negative (1)

Table VI: Similarity matrix of the strains based on Jaccard index

	EB1	EB2	EB3	EB4	EB5
EB1	1	0.14	0	0.14	0.14
EB2	0.14	1	0.14	0.14	0.28
EB3	0	0.14	1	0	0.14
EB4	0.14	0.14	0	1	0.14
EB5	0.14	0.28	0.14	0.14	1

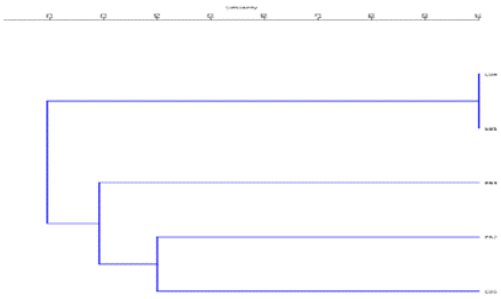

Fig 4: Hierarchical clustering with UPGMA algorithm

2.17. Effect of metal exposure

No mortality was observed among any treated group of earthworms. But the number of gut microflora varied in different exposures which have been summarized below in Table VII.

The data obtained from the CFU calculation for each strain were then compared with the control (Fig 5).

Table VII: Summarized effects of both the doses of metal on the isolates from *Eisenia fetida*

Isolates	Abundance/Presence/Absence		
	Control	MDI	MDII
Bacillus cereus	++++	++	_
Clostridium sp.	+++	+	+
Vibrio comma	++++	+	+
Bacillus subtilis	++++	+	_
Staphylococcus sp.	++++	+++	++
Penicillium notatum	++++	+++	+
Penicillium sp.	++	+	+
Ulocladium sp.	++	-	-
Exophiala werneckii	++	++	-
Candida albicans	+++	+	-

+ Presence; ++ Average growth; +++/more Abundance; - Absence

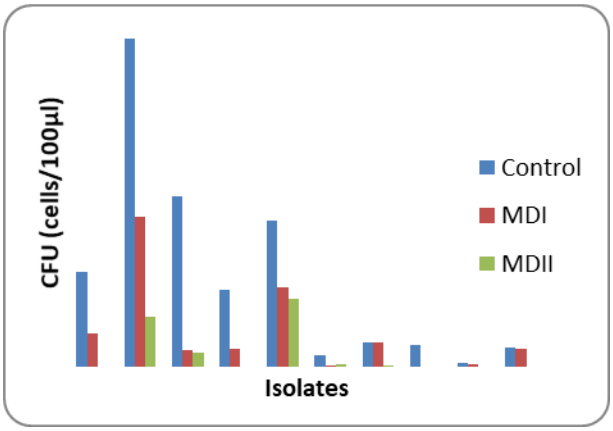

Fig 5: Bar diagram showing the effect of the two doses of Metal zinc (MDI and MDII) on the bacterial and fungal isolates of *Eisenia fetida*

2.18. Effect of pesticide exposure

No mortality was observed among any pesticide treated group of earthworms. But the number of gut microflora varied in different test conditions as summarized below in table VIII. Also the comparison of effect has been shown in Fig 6.

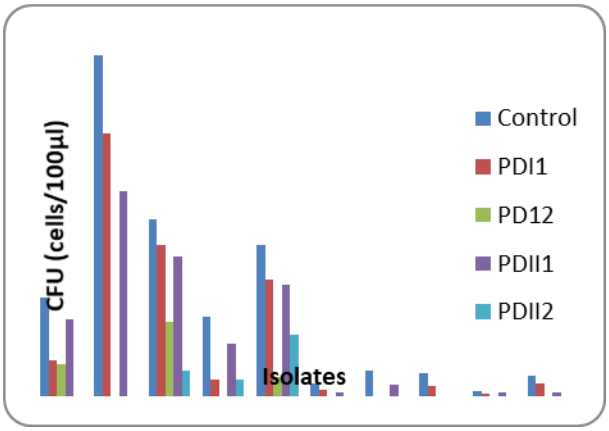

Fig 6: Bar diagram showing the effect of the pesticides (PI and PII) on the bacterial and fungal isolates of *Eisenia fetida*

Table VIII: Summarized effects of both the doses of pesticides on the isolates from *Eisenia fetida*

Isolates	Abundance/Presence/Absence				
	Control	PI1	PI2	PII1	PII2
Bacillus cereus	++	+	+	+	-
Clostridium sp.	++	++	-	++	-
Vibrio comma	++++	+++	++	+++	++
Bacillus subtilis	++	+	-	++	+
Staphylococcus sp.	++++	+++	+	+++	++
Penicillium notatum	++++	++	-	++	-
Penicillium sp.	+++	-	-	+	-
Ulocladium sp.	++	++	-	-	-
Exophiala werneckii	++++	+	-	+	-
Candida albicans	++	+	-	+	-

+ Presence; ++ Average growth; +++/more Abundance; - Absence

4. DISCUSSION

Earthworms are known to play important roles in the physical and chemical properties of soil. Once consumed, organic materials may be subjected to digestive enzymes produced by the earthworms or organisms living in their intestines. This understanding led to the assumption that the gut intestine of earthworms may contain a diverse community of microorganisms that are most likely living in symbiotic- and/or associative-type relationships. Thus, in this study, the culturable microbial community structure of the earthworm gut (*Eisenia fetida*) was analyzed.

An observation made for the specie *Eisenia fetida* coming from contaminated soil in an industrial zone ; the genus *Bacillus* was the dominant group found in the intestines of the earthworms [31]. The earthworms for our study were bought from Mecheda, West Bengal, which is a place closed to the Kolaghat Power plant, West Bengal, so, the soil from which these worms were collected might be contaminated as out of five identified bacterial isolates, two are of the genus *Bacillus*. Bacterial species from some genuses like *Azospirillum, Acetobacter, Bacillus* and *Vibrio* have also been reported which is a little similar with our findings[32, 33]. Fungal species, such as *Aspergillus sp. Fusarium sp., Gliocladium sp., Penicillium sp.* and *Trichoderma sp.*, have been reported by Parthasarathi *et al*., 2002 [34].

Also, we observed the effect of the metal Zn^{2+} and two commonly used pesticides Pendimethalin and Chlorpyrifos on these gut microflora of earthworm *Eisenia fetida*. The fungal isolates were absent in the higher dose of the pesticides but the bacterial isolates were present despite they reduced in their colony number. Only the fungus with genus *Penicillium* survived the higher dose of the metal contamination. Overall, the fungal species were mostly affected by the contamination in the soil. Taking into consideration the role of the earthworm in bioremediation, we should consider those microbes that are surviving even at higher doses of the contaminants. These microbes might have a mechanism to sustain the pollutants in the soil and thus can be the promising soldiers for bioremediation. But such conclusions need a more detailed study.

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