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ELUCIDATION OF INTERACTION BETWEEN EARTHWORM AND MICROBIAL HABITAT

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ABSTRACT

In the present study, four groups (normal, sterile, antibiotics & antifungal agent and 2% agar) of organic media were used for the analysis of physiological and mortality of earthworms which were inoculated into their respective media. The worms in the sterile media died within three days of incubation followed by the worms present in antibiotics and antifungal added media, where 100% mortality occurred within four days of incubation. In agar media, the results were similar to antibiotics and antifungal added media where it produced 100% mortality within four days of incubation. Similarly, cocoon inoculated in sterile media failed to hatch where its counterpart in normal media got hatched within five weeks. A rescue assay was attempted to rescue the worms in the sterile media by partially replacing the last micro biota. But this strategy failed to rescue both the worms and the cocoons. The gut micro biota analysis between the worms present in the sterile and normal media did not produce any difference in the microbial population. Finally, the skin micro flora role in the mortality of the worms was analyzed. The worms were treated externally and internally with different antibiotics in which the worms treated with tetracycline produced 100% mortality within two days.

INTRODUCTION

Earthworms are thought to be the most ancient soil animal having started colonizing terrestrial environments about 600 million years ago[1]. Earthworms are semi-aquatic animals which extract water continuously from the surrounding environment in order to maintain their cuticle in a moist state to facilitate respiration. Worm castings or vermicast contain organic particles in fine sizes. Some of the earthworm's mucus is excreted and mixed with the vermicasts favoring microbial activity. The high content of ammonia and organic matter in partial stage of decomposition in vermicasts further provide nutrients which promote microbial growth and high rate of decomposition[2]. Earthworms are the most important ecosystem engineers (organisms that may modify or create their habitat and thus influence availability of resources to other species and soil properties) in arable soil due to their lasting effects on soil physical and biochemical properties[3].

The *Eudrilus eugeniae* species of earthworm belongs to the Eudrilidae; it is a native African species that lives in both soils and organic wastes but has been bred extensively in the United States, Canada and elsewhere for the fish-bait market, where it is commonly called the African night crawler. It is a large, robust earthworm that grows extremely rapidly and it is relatively prolific when cultured. Under optimum conditions, it could be considered an ideal species for animal feed protein production. Its main disadvantages are a relatively narrow temperature tolerance and some sensitivity to handling. *Eudrilus eugeniae* can live in soils and has high reproduction rates[4,5]. It is capable of decomposing large quantities of organic wastes rapidly and incorporating them into the topsoil[6,5,4]. The life cycle of *Eudrilus eugeniae* ranges from 50 to 70 days and its life span can be 1 to 3 years.

Eudrilus eugeniae is a purplish red worm. It is detritivores as they feed on the soil surface and mostly the plant litter. It grows rapidly and is quite prolific but is very sensitive to changes in their environment. *Eudrilus eugeniae* grows well at a temperature of more than 25°C but best at 30°C[7] attaining maximum weight, length and number of segments in about 15-20 weeks[8]. The length ranges from 111-300 nm and size may depend on habitat[9]. It has a purple sheen and the posterior segments are evenly tapered to a point[10]. It has sigmoid shaped setae. It displays a perichaetine arrangement where the setae gets arranged in a ring right around the segment with a large or small break in the mid dorsal and mid ventral regions[11]. Different species of earthworms have different life histories occupying different ecological niches and have been classified on the basis of their feeding and burrowing strategies into three ecological categories: epigeic, anecic and endogeic[12]. Endogeic (soil feeders) and anecic species (burrowers) live in the soil and consume a mixture of soil and organic matter and thus excrete organ-mineral faeces. Epigeic species of earthworms are litter dwellers and litter trans-formers; they live in organic soil horizons in or near the surface litter and feed primarily on coarse particulate organic matter.

Most studies of the microbial contribution to soil processing have focused on the gut and casts (faecal pellets) employing both culture based and more recently, molecular methods[13,14]. We are investigating a less familiar association of a dense culture of bacteria in the nephritis, excretory organs present in each segment of the earthworm. Although discovered around 1926, the identity and the activity of these bacteria within the worm had not been extensively investigated. Acidovorax-like bacteria have been isolated from the nephritis of the compost earthworm *Eisenia fetida* and a systematic description of the symbiotic organisms is being conducted[15]. Some microorganism species were submitted to growing stimulation during gut transit[16]. Indeed, the survival of microorganisms in the earthworm gut depends on their capacity to resist to digestive enzymes of microbial or earthworm origins, intestinal mucus, $CaCO_3$ or to bacteriostatic and microbial substances[17]and also transit time[18]. Analysis of the digestive tract contents of earthworms has revealed the presence of grass fragments and other plant leaves, roots, algal cells, seeds, fungi, bacteria, protozoa and actinomycetes[19].

The microbial composition of earthworm intestine contents has been considered to reflect the composition of the soil or ingested plant remains[20,17,21] but there is evidence of the possible existence of ecological group-specific gut micro biota in some earthworm species[1]. Indeed, some physical links were found between bacterial cells and epithelium in the hindgut of *L. terrestris*[22]. The presence of a mutualistic digestive system was demonstrated in several tropical and temperate earthworm species in which soluble organic carbon, in the form of a mixture of low-molecular weight mucus was added to enhance the soil microflora proliferation[23,24]. Intestinal mucus is composed of amino acids (about 200 Da) mixed with high-molecular weight sugars and glycoproteins (40,000-60,000 Da)[25]. The mucus production and the enzyme pool depend on earthworm species and food quality. For example, epigeic species which feed on rich substrates need a complex enzymatic system but not an intensive mucus production in their gut[24]. Moreover, earthworm gut was identified as an ideal habitat for N2O producing bacteria because earthworms activate these microorganisms during gut passage[26,27]. However, a comprehensive description of the digestive system and the origin of different gut enzymes require further research, particularly for epigeic and anecic species[28].

An increasing appreciation of the synergistic interactions between earthworms and microorganisms is observed. The main interest is focused on microorganisms that are ingested from soil and transit the gut by employing culture-based and molecular methods[29,30,31,32]. Despite those recent studies, the real existence of symbioses in the earthworm gut is still controversial[33]. Reports are available that microbial fingerprints in the earthworm gut are associated to the microbial profile in soil and in food sources[34,35,36,37]. For instance, only small differences in bacterial communities between soil, gut and fresh casts of *L. terrestris* have been highlighted suggesting the existence of an indigenous earthworm microbial community as unlikely[29]. Similar results were found by Knapp *et al*. (2009)[38] during their study on the impact of a radical diet shift on gut micro biota of *Lumbricus rubellus*[37]where all the actinomycetes isolated from the casts occurred in the natural soil of their study. However, other studies show some evidence of earthworm gut symbionts[39,31]. They found some microorganisms in the earthworm intestine that are absent in the surrounding soil[31] and important changes in the fatty acid concentration and composition in the gut of the earthworm *L. terrestris*[39].

The aim and objectives of the study is focused on to elucidate the earthworm and microbe interaction in its habitat, the earthworm cocoons and microbe interaction in its habitat.

MATERIALS AND METHODS

Preparation of compost bedding and inoculation of earthworms

The compost bedding was prepared with leaf litter and cow dung in a small cement tank. A layer of leaf litter was placed as a basal layer of thickness at least 6.5-7.5cm, over this, a layer of cow dung to a thickness of at least 15-16 cm was added and then the layers are moistened carefully to avoid stagnation of water[40,41,42]. About 10-15 earthworms (*Eudrilus eugeniae*) were inoculated to the bedding followed by the addition of small lumps of cow dung over the soil[43]. The moisture level of the media was maintained between 30-40%. By the end of the month, adult earthworms were noticed.

Collection of earthworms

Earthworms were collected from the compost bedding of small cement tank in the Vermibiotechnology Laboratory, Department of Biotechnology, Manonmaniam Sundaranar University, Tirunelveli. About 100 sexually matured clitellated earthworms (*Eudrilus eugeniae*)[44,45,46] were collected and washed in running tap water.

Gut clearance of earthworms *Eudrilus eugeniae*

About 0.5g of agar was accurately weighed in a beaker and dissolved in 100ml of distilled water by melting it for complete dissolution. Earthworms were introduced into the solidified agar media and the beaker was covered with nylon net to avoid escape of the worms and kept overnight[47].

Physiological and mortality analysis of earthworm *Eudrilus eugeniae*

Eudrilus eugeniae was cultured in four different media for the physical and mortality studies.

Group 1: About 250 g of organic medium was taken in a glass bottle.

Group 2: About 250 g of the organic medium was taken in a glass bottle and sterilized at 15 lbs. pressure for 15 minutes and cooled to room temperature.

Group 3: About 250 g of the organic medium was taken in a glass bottle and treated with antibiotics (Chloramphenicol, Tetracycline) and antifungal agent (Griseofulvin).

Group 4: 2% agar was prepared in a glass bottle, sterilized under pressure and cooled to room temperature.

Previously weighed five earthworms were added to each groups and the mouth of the glass bottles was covered with aluminum foil to avoid further contamination. The weight and mortality of these earthworms was noted upto five days[48,49].

Enumeration of microbes from soil and gut of earthworm *Eudrilus eugeniae*

One g of soil sample from all the three sets of medium (normal, sterile, antibiotics and antifungal agent) were serially diluted upto10^{-6} dilution and 0.1 ml of the diluents was plated onto nutrient agar plates and potato-dextrose agar plates by spread plate technique. Highest dilutions (10^{-4} and 10^{-6}) were selected for non-sterile media and lowest dilutions (10^{-2} and 10^{-4}) were selected for sterile as well as antimicrobial agent treated media. One earthworm from each group was dissected out and the content from the gut were removed[50,51,52]. 0.1g of the gut content was serially diluted up to 10^{-6} dilution and 1 ml from each dilution were plated on to sterile nutrient agar plates and incubated for 24 hours in an inverted position. After 24 hours of incubation, the number of colonies present in each dilution was calculated.

Hatching of cocoons

Cocoons were collected from vermin bed and surface sterilized. About 250 mg of organic medium was taken in a glass bottle into which 10 cocoons were introduced and labeled as normal. About 250 mg of organic media taken in a glass bottle was sterilized into which 10 cocoons were introduced and was labeled as sterile. In another glass bottle, organic media and 250 mg of antibiotic (Chloramphenicol, Tetracycline) and antifungal agent (Griseofulvin) were taken and mixed together into which 10 cocoons were added and marked as antibiotics and antifungal agent. The inoculated cocoons were observed for 3 to 5weeks.

Rescue assay of earthworm *Eudrilus eugeniae*

One gram of soil sample was taken from vermin bed and was inoculated into 10 ml of sterile nutrient broth and incubated at 37°C for 24 hours. Four sets of sterile organic medium were prepared and to two sets, five worms were introduced and to another two sets, cocoons were introduced. 10 ml of nutrient broth was added to each set separately and incubated and the biomasses of the earthworms were noted for a period of three days and the hatching of cocoons were noted for 21 days.

Response of earthworm against antibiotics and antifungal agents

Four earthworms were taken and subjected to surface sterilization. Each earthworm was injected with antibiotics (Chloramphenicol, Tetracycline and Ampicillin) and antifungal agent (Griseofulvin) separately in a concentration of about 2.50 mg/ml in the clitella region and introduced into normal organic media and the results were noted for five days. Five earthworms were taken and different concentrations of Tetracycline (0.75 mg, 1.25 mg, 2 mg, 2.25 mg and 2.50 mg) were prepared and injected in the clitellum region and the earthworms were introduced into normal organic medium and the results were noted. Three earthworms were taken and was surface sterilized with tetracycline antibiotic and introduced into normal organic medium, tissue paper and 2% agar and incubated for seven days and the results were recorded.

RESULTS AND DISCUSSION

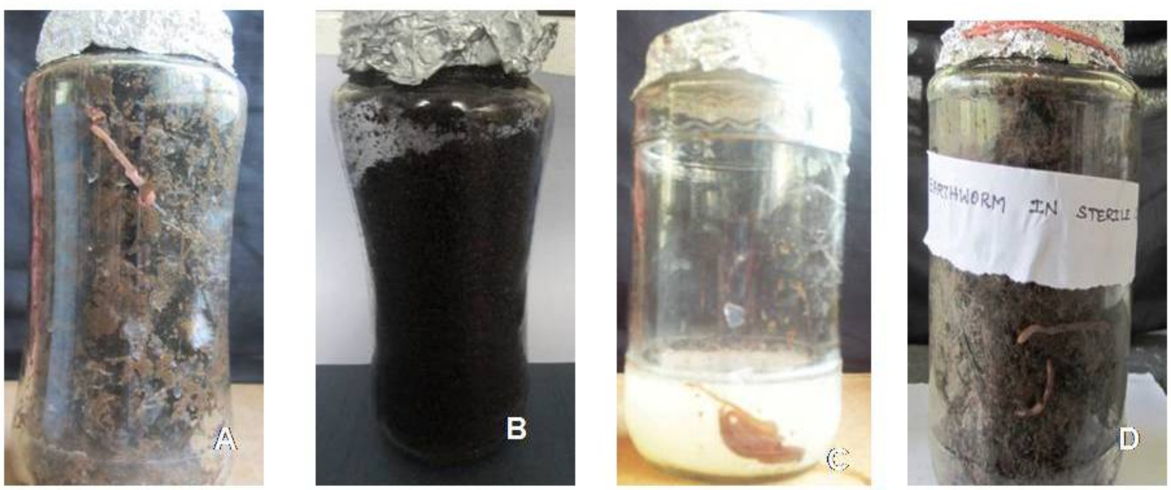
Townsend *et al*. (2004)[53]stated earthworms play a vital role in the soil fertility and were described as“natures plough’’ by Charles Darwin. These ecological engineers affect the physical and chemical properties of the soil by degrading the leaves and other organic wastes and mix them with the soil was reported by Jimenes *et al*[54]. Mostly a single species of earthworm is employed for vermicomposting process of leaf litter and cattle manure. These worms are in symbiotic association with protozoans, fungi, actinomycetes and bacteria, thereby control the soil ecosystem.

Physiological and mortality analysis of earthworm *Eudrilus eugeniae*

Earthworms were cultured in four different groups of medium (Figure 1 & 2) and the biomass of earthworms from each group was taken up to eight days and the results were tabulated (Tables 1-4). In normal organic medium, the biomass of earthworms oscillated throughout the experimental period (Graph 1) whereas the earthworm biomass in the sterile organic medium was observed to decrease rapidly from day 1 to day 2 (Graph 2). In organic medium with antibiotics and antifungal agent, the earthworm biomass decreased gradually up to day 4 (Graph 3 & 4).

Figure 1: Earthworm *Eudrilus eugeniae* – Test animal

Figure 2: Four groups of organic medium to assess mortality of *Eudrilus eugeniae*



A-Normal organic medium; B-Organic medium with antibiotics and antifungal agents; C-2% agar medium; D-Sterile organic medium

TABLE 1: BIOMASS OF EARTHWORMS IN NORMAL ORGANIC MEDIUM

Number of days	Biomass of *Eudrilus eugeniae* in normal organic medium (g)					Average biomass (g)
1	0.652	0.434	0.291	0.497	0.379	0.4506
2	0.685	0.308	0.231	0.432	0.157	0.3625
3	0.249	0.268	0.772	0.245	0.484	0.4028
4	0.456	0.189	0.268	0.177	0.577	0.3294
5	0.293	0.133	0.185	0.209	0.163	0.1966
6	0.2	0.18	0.51	0.31	0.25	0.290
7	0.2	0.24	0.19	0.18	0.42	0.246
8	0.36	0.03	0.09	0.22	0.06	0.152

TABLE 2: BIOMASS OF EATHWORMS IN STERILE ORGANIC MEDIUM

Number of days	Biomass of *Eudrilus eugeniae* in sterile organic medium (g)					Average biomass (g)
1	0.58	0.92	1.1	1.11	0.68	0.878
2	0.07	0.13	0.31	0.23	0.34	0.153
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	0	0	0	0	0	0
8	0	0	0	0	0	0

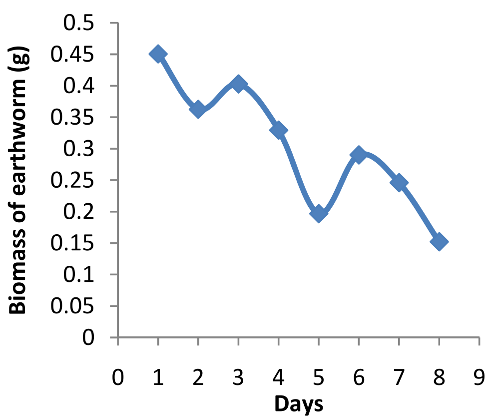
TABLE 3: BIOMASS OF THE EARTHWORM IN 2% AGAR MEDIUM

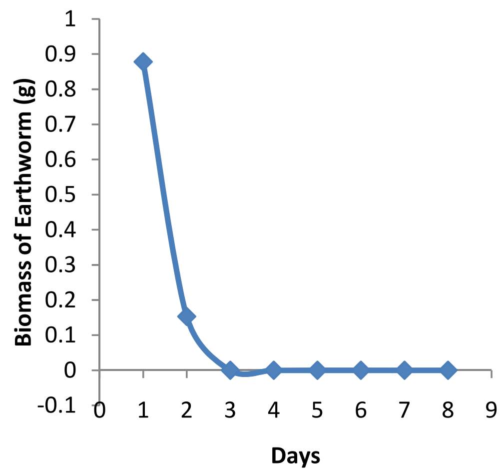
Number of days	Biomass of *Eudrilus eugeniae* in 2% agar medium (g)					Average biomass (g)
1	0.29	0.298	0.234	0.725	0.666	0.442
2	0.19	0.2	0.16	0.21	0.23	0.198
3	0.21	0.2	0.17	0.1	0.14	0.164
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	0	0	0	0	0	0
8	0	0	0	0	0	0

TABLE 4: BIOMASS OF THE EARTHWORM IN ORGANIC MEDIUM WITH ANTIBIOTICS AND ANTIFUNGAL AGENTS

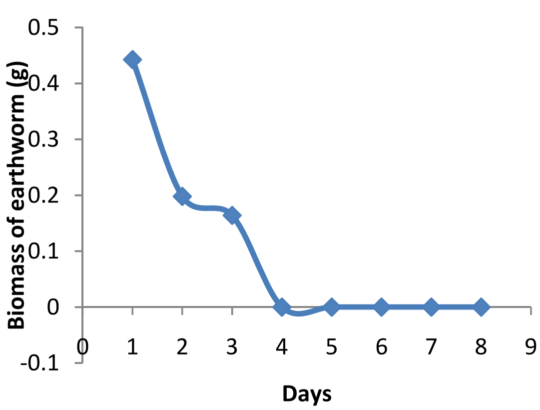
Number of days	Biomass of *Eudrilus eugeniae* in organic medium with antibiotics and antifungal agents (g)					Average biomass (g)
1	0.58	0.92	0.43	0.84	0.68	0.4
2	0.077	0.139	0.314	0.54	0.43	0.46
3	0.074	0.051	0.073	0.41	0.24	0.2
4	0.031	0.043	0.034	0.27	0.18	0.16
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	0	0	0	0	0	0
8	0	0	0	0	0	0

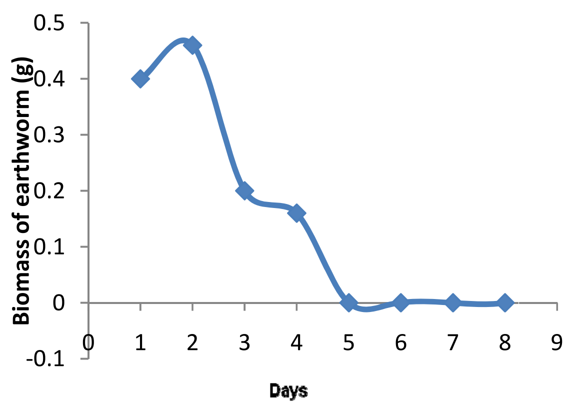
Graph 1 & 2: Showing biomass of earthworm in normal and sterile organic medium





Graph 3 & 4: Showing biomass of earthworm in oraganic medium with antibiotics & antifungal agent and 2% agar medium





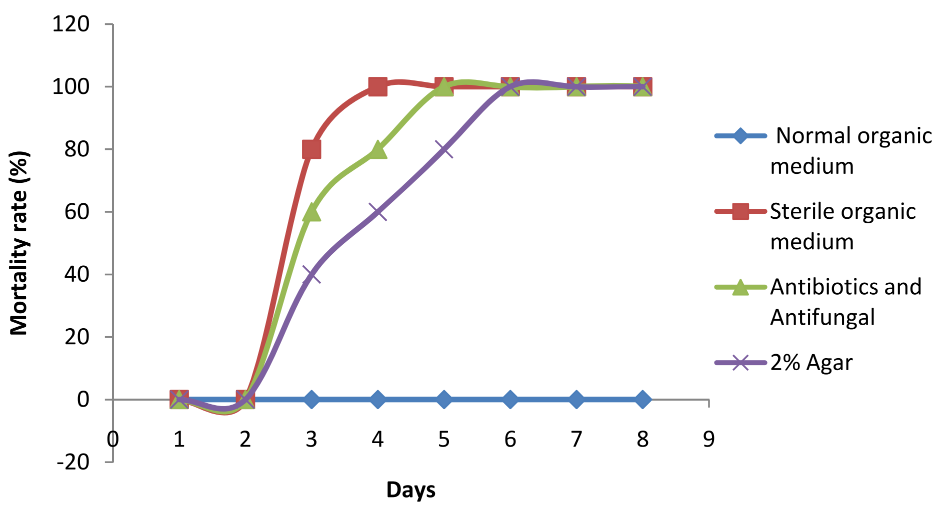
The mortality rate of earthworms was evaluated in three groups of medium (normal organic medium, sterile organic medium, organic medium with antibiotics and antifungal agents). The earthworms cultured in normal organic medium were alive up to eight days, but the mortality rate of earthworms in sterile organic medium, organic medium with antibiotics and antifungal agents was 100% with variations in time. The earthworms died within third day in sterile organic medium and within five days in organic medium with antibiotics and antifungal agents (Table 5 & Graph 5).

TABLE 5: MORTALITY RATE OF EARTHWORMS IN THREE GROUPS OF ORGANIC MEDIUM

Number of days	Mortality rate %			
	Normal organic medium	Sterile organic medium	Organic medium with antibiotics and antifungal agents	2% agar medium
1	0	0	0	0
2	0	0	0	0
3	0	80	60	40
4	0	100	80	60
5	0	100	100	100
6	0	100	100	100
7	0	100	100	100
8	0	100	100	100

0: No mortality occurs in days and 100: mortality occurs.

Graph 5: Showing mortality rates (%) of earthworms in four groups of medium



Highest mortality of earthworms was noted when cultured in sterile medium followed by agar and antimicrobial agent treated media. No mortality was noted in normal group where no changes were induced in the culture medium. A slight reduction in the weight was observed during the first day of the experiment which might be due to the time taken to get acclimatized for the earthworms. All earthworms died on the day 3 when cultured in the sterile medium. Complete mortality was observed on the day 5 in the rest of the experimental groups. A drastic weight loss was observed in all experimental groups except normal (Group I). Meena Khwairakpam and Renu Bhargava (2007)[55] also reported the reduction in earthworm biomass in a composting analysis with three different earthworms *Eisenia fetida, Eudrilus eugeniae, P. excavatus* both individually and in co-culture methods. Earthworm biomass was reported to be increased in all four experimental groups as in the normal group in the present study.

The microbes which were present in the soil of normal organic medium, sterile organic medium and organic medium with antibiotics and antifungal agent was enumerated and the results were tabulated (Table 6).

TABLE 6: MICROBIAL COUNT OF SOIL IN THREE GROUPS OF ORGANIC MEDIUM

Medium used	Dilution factor	Number of colonies (CFU/ml)						
		Number of days						
		1	2	3	4	5	6	7
Normalorganic medium	10^{-4}	19	98	45	TNTC	TNTC	TNTC	TNTC
	10^{-6}	15	36	35	48	TLTC	TLTC	TLTC
Sterile organic medium	10^{-2}	0	2	8	-	-	-	-
	10^{-4}	0	0	0	-	-	-	-
Medium with antibiotics and antifungal agents	10^{-2}	36	72	120	-	-	-	-
	10^{-4}	10	4	23	-	-	-	-

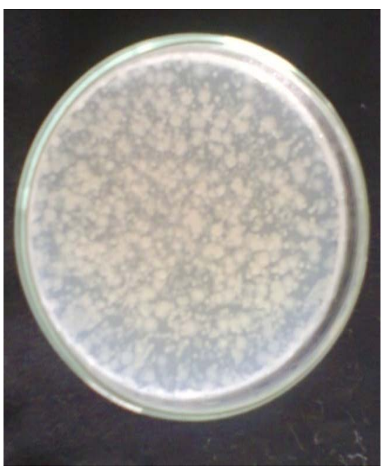
TNTC: Too Numerous To Count; TLTC: Too Low To Count

Table 6 shows the enumeration of bacteria in the culture medium during the experimental period. Normal group possessed numerous bacteria as it didn't undergo anykind of disinfection and sterilization. Sterile medium showed the presence of few bacteria in the day 2 and 3. This may be due to cross contamination during handling or by the growth of symbiotic bacteria from the gut excreta or external surface of the earthworm. Presence of bacteria in the third group indicates the selective clearing of bacteria by antimicrobial agents.

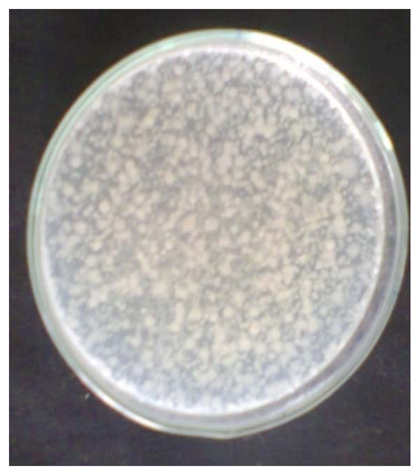
Agar medium is devoid of nutrient content and prevents the growth of any kind of microbes and hence remained sterile until the completion of the experiment. Furlong *et al*., (2002)[13] reported that the composition and structure of microbial populations of the earthworm's digestive tract have shown that some microorganisms of soil (*e.g.Pseudomonas* sp. and *Firmicutes* sp.) increase in abundance through the gut tract of *L. rubellus*. Automated image analysis and *in situ* hybridization were used to study the gut transit impact on bacterial community structure.

The gut microbes which were associated with the earthworms present in normal and sterile organic medium were isolated. The microbial count in the gut microbes in the earthworms in normal and sterile organic medium was found to be same (Plate 2). According to Thakuria *et al*., (2010)[32] the development of distinct gut wall-associated bacterial communities is strongly associated to earthworm ecological group, despite the shift observed with food source and habitat changes. Presence of all bacteria in earthworm gut and in soil does not allow determination of whether the bacterial communities share a symbiotic or a mutualistic metabolic interaction with earthworms.

Plate 2: Microbes isolated from the gut of *Eudrilus eugenia*



A-Normal organic medium **A**



B-Sterile organic medium **B**

Hatching of cocoons of earthworm

Cocoons were cultured in two different groups of medium (normal organic medium and sterile organic medium). In normal organic medium, the cocoons got hatched on fifth week (Plate 3) and the cocoons cultured in sterile organic medium get shrined and died within two weeks (Plate 4).

Plate 3: Hatched cocoons in normal organic medium

A: Cocoons in normal organic medium

A

B: Juveniles

B

Plate 4: Hatched cocoons in sterile organic medium

A

A: Cocoons in sterile organic medium

B

B: Shrinkage of cocoons

Rescue assay of earthworm *Eudrilus eugeniae*

One gm of soil sample was taken from vertices and inoculated in 100 ml of nutrient broth and incubated. After incubation, the 10 ml inoculums were mixed with sterile organic medium which contained earthworm and cocoons. The earthworms died and cocoons got shrined in two days of incubation (Plate 5).

Plate 5: Earthworms and cocoons cultured in sterile organic medium inoculated with soil cultures

A

A:Dead earthworms

B

B: Shrinkage of cocoons

Response of earthworms against antibiotics and antifungal agent

The earthworm was injected with antibiotics (Chloramphenicol, Tetracycline and Ampicillin) and antifungal agent (Griseofulvin). The earthworm injected with tetracycline in a concentration of 125 µg/ml died within two days. Different concentrations of tetracycline (25 µg, 50 µg, 100 µg and 125 µg/ml) were injected into the clitella of five earthworms in which all the earthworms died within three days irrespective of the concentration. Three earthworms were surface sterilized with tetracycline and introduced into normal organic medium tissue paper and 2% agar in which the earthworm was alive in normal organic medium and 2% agar, but bleeding was observed in the clitellum region of the earthworm when cultured in tissue papers (Plate 6). Anjakotzerke *et al.*,[57] reported that the manure contaminated with the antibiotic, Sulfadiazine impairs the abundance of nirK-type and nirS-type denitrifies in the gut of the earthworm, *Eisenia fetida*. The antibiotic Sulfadiazine (SDZ) can affect denitrifying bacteria in soil. However, effects on denitrifies in the gut of earthworms have not been described so far. Therefore, the influence of SDZ-contaminated manure applied to soil on denitrification in the gut of the earthworm, *Eisenia fetida* was assessed by quantitative polymerase chain reaction targeting genes coding for nirK-type and nirS-type nitrite reductases of denitrifies. Gut contents of *Eisenia fetida* contained 2.5×10^6 and 5.1×10^5 gene copies of nirK and nirS, respectively, after two weeks in soils amended with manure only. Copy numbers of nirK and nirS in gut contents from manure treatments with SDZ were up to ten times less. Overall, the data indicate a negative impact of SDZ on denitrifies in the gut of earthworms.

Plate 6: Earthworms surface sterilized with tetracycline and cultured in tissue paper and 2% agar medium

A

A: Bleeding of earthworms

B

B: Alive earthworm in 2% agar medium

CONCLUSION

It is observed from the present investigation that, the earthworms prefer moist environment for the active surface respiration and hence lack of moisture may lead to the death of earthworms due to severe dehydration. Moisture content of all the organic media was thoroughly maintained throughout the experimental period; hence lack of moisture can never lead to the death of *Eudrilus eugeniae*. The nitrogen and organic content of all the media were same except in the 2% agar medium and it is assumed that these factors may not involve in the death of earthworms. When the media is sterilized or disinfected, the microbial equilibrium will get destroyed. This may lead to the disruption of the symbiosis, change the physical as well as biological characteristics of the culture media and break the food chain ultimately leading to the death of earthworms and replacement of substrate with unwanted microbes. The pH of the substrates also affects the growth and survival of *Eudrilus eugeniae* and quality of compost produced. The microbes in the environment of *Eudrilus eugeniae* decrease the pH of the substrates to an optimum for the maximum productivity and survival of the organism. An environment devoid of favorable microbes might have prevented the modification of initial substrate pH to the favorable alkaline pH. This might have led to the death of earthworms in the sterile or disinfected media. It is clear that the withdrawal of nutrients led to the death in the group IV with 2% agar substrate.

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