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Subinhibitory Levels of Oxytetracycline in Earthworm Meal Significantly Boost Resistance-Mutation Rates in *Bacillus* spp. within the Gut of *Eisenia fetida*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To estimate changes in the number of oxytetracycline -resistant strains, a dynamic population of *Bacillus* in the gut of *Eisenia fetida* cultured in processed-cow-dung (PrCD) supplemented with subinhibitory amounts of oxytetracycline, was studied.
Study Design: Sterile water containing oxytetracycline was sprayed over PrCD at concentration of 0.012 µg per mg dehydrated PrCD per spraying. The number of oxytetracycline-resistant *Bacillus* spp. microbiota of *E. fetida's gut* was compared to the number of oxytetracycline-resistant *Bacillus* spp. in its feed (PrCD). For this purpose, Luria Agar (LA) and Bacillus Agar (BA) plates amended with or without oxytetracycline were used.
Place and Duration of Study: The study was carried out in the Omics Laboratory of Department of Biotechnology, University of North Bengal situated in Darjeeling district of West Bengal, India during 2017-18.

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Methodology: *E. fetida* gut content and PrCD samples were collected on different days of the experiment for bacteriological analysis. Dilution plating on LA yielded the total number of cultivable heterotrophic bacteria. LA plates amended with oxytetracycline (15 µg/ml) were used to acquire the fraction of heterotrophic bacteria resistant to oxytetracycline. BA plates were also utilized to obtain the fraction of resistant *Bacillus* spp. population. The frequency of mutation was determined using a conventional formula.

Results: In the gut of *E. fetida* reared in PrCD supplemented with sub-inhibitory concentration of oxytetracycline, a rise in the oxytetracycline-resistant *Bacillus* population was observed. On day 1, the frequency of oxytetracycline-resistant *Bacillus* spp. (5×10^{-8}) matched the spontaneous mutation frequency, however higher frequencies on days 2 and 7 (1.6×10^{-4} and 3.5×10^{-6} respectively) suggested significant dissemination of oxytetracycline resistance in the gut environment.

Conclusion: Subinhibitory oxytetracycline concentrations in earthworm diet had a significant effect on mutation rates, showing that evolutionary forces on the gut microbiota may be determining their responses to antibiotic stress.

Keywords: Oxytetracycline; Eisenia fetida; sub-MIC; Bacillus sp.; gut microbiota; antibiotic resistance.

1. INTRODUCTION

Antibiotic resistance (AR) has evolved and spread many harmful bacteria as a result of improper and unregulated antibiotic use [1]. AR is spreading at an alarming rate, posing a major threat to not only humanity but the entire animal kingdom, as resistant populations are difficult to treat with the antibiotics currently available. It is critical to determine the most common mechanisms by which microbial species become resistant, as well as the rate at which they do so [2,3]. *Bacillus* populations grow resistant to the exposed antibiotic and evolve very quickly in real time under controlled laboratory circumstances with dynamic transient passages through the stomach of the dung-eating worm *Eisenia fetida* [4]. Because *E. fetida* is an epigeic earthworm, it is rarely seen in the subsoil. Cow dung can help it flourish since it contains both organic matter and microorganisms that it needs. When bacteria found in processed cow dung (PrCD) are exposed to antibiotics at 'lower-than-inhibitory concentrations', it aids their adaptation to a new environment with less antibiotic presence. The worm gut contains hundreds of different bacteria, with the Firmicutes being one of the most common; they are frequently released (mixed in with the cast) into the environment. As a result, *E. fetida* could be classified as a detritivorous organism. In the laboratory, PrCD functions as both a habitat and a food source. Different *Bacillus* spp. are mostly detected in the gut of *E. fetida* among the Firmicutes [5]. It's been reported that some *Bacillus* species have genes that make them resistant to a variety of antibiotics [6].

AR development is a widespread phenomenon that has been identified as a key source of concern. AR is caused by the existence of certain genes in chromosomesl or extrachromosomal genetic elements in many species, as well as efflux proteins, which pump antibiotics out of the system, and enzymes that they create to inactivate antibiotics [7]. Antibiotic-resistant genes have been reported to be transferred horizontally from antibiotic-resistant microbes to antibiotic-sensitive bacteria. Oxytetracycline, a member of the tetracycline antibiotic family, is extensively used to treat acne in humans, and a significant proportion of it is used in bovine feed to treat bacterial infections. Some *Bacillus cereus* strains have been found to be resistant to oxytetracycline in previous research [8-9]. *Bacillus cereus* has β-lactamase activity [10], as well as tetracycline and oxytetracycline resistance, which was discovered subsequently [11]. Antibiotics at sublethal or subinhibitory quantities are frequently exposed to bacteria. This happens in the environment as a result of agricultural operations and wastewater treatment and run-off, as well as in the clinic as a result of inappropriate therapy, poor adherence, and low-quality drugs [12-14]. Although subinhibitory doses do not kill bacteria, they can cause stress and put bacteria under selection pressure. A few studies on subinhibitory antibiotic exposure and its impact on bacterial resistance are currently available [15-18]. In the current study, bacteria resistant to oxytetracycline were quantified in both inhibitory and subinhibitory concentrations of the antibiotic in the gut and feed of *E. fetida*, as well as to see if the challenge of a subinhibitory dose of oxytetracycline in the worms' feed (PrCD) affects the occurrence of oxytetracycline-resistant strains. The overall aim of this work was to quantify fold changes of mutation rate in *Bacillus* spp. population of *E. fetida*'s gut on subinhibitory

oxytetracycline exposure via feed relative to an unexposed control group.

2. MATERIALS AND METHODS

2.1 Rearing of *Eisenia fetida* on PrCD Feed

Adult *E. fetida* specimens were obtained from the University of North Bengal's Centre for Floriculture and Agro-business Management (COFAM) in Darjeeling. The worms were kept in plastic tubs in the laboratory with dried cow dung at 22°C and 70-80% humidity [19]. Cow dung samples from healthy cows (*Bos taurus indicus*) were collected and processed into chips, then sun-dried for two days in the open air (8 h per day) to produce semi-dried, odourless chips. The chips were broken into small pieces, sprayed with sterile distilled water to moisten them to 70% moisture content, and then placed in an incubator at 25°C for 48 hours to create the bed for subsequent tests, 100 g of this PrCD was placed in a sterile Petri-plate (diameter = 14 cm). In each of the PrCD-containing plates, twenty-five adult *E. fetida* earthworms (weighing 0.3- 0.5 g each) were reared.

2.2 Antibiotic Treatment of the Earthworm Feed

Three sets of distinct studies in triplicate for each antibiotic were designed for subinhibitory exposure of oxytetracycline. The antibiotic oxytetracycline had a stock concentration of 15 mg/ml. Every day, antibiotic-containing water was sprayed on the PrCD bed in the experimental set-up. To do this, we mixed 80 µl of oxytetracycline (from the stock solution) with 10 ml of sterile water and sprayed it on the PrCD experimental plates to maintain a moisture level of 75% (±5%). Assuming that all antibiotics molecules in solution state get adsorbed to compost particles, the concentration of oxytetracycline would be 0.012 µg per mg of dehydrated compost, imposing ~~a~~ subinhibitory antibiotic stress to the compost-microbiota.

2.3 Monitoring of Cultivable Bacterial Load and Major *Bacillus* spp. Diversity in PrCD with Assessment of Mutational Frequency

The PrCD plates used in the studies were made up of five distinct combinations of components (in each combination PrCD remained as a constant constituent). The following is a description of the experimental setup: (i) plate containing 25 mature *E. fetida* in oxytetracycline-supplemented PrCD, (ii) plate containing solely oxytetracycline-supplemented PrCD (without *E. fetida*), (iii) PrCD without antibiotics and *E. fetida*, and (iv) PrCD with 25 mature *E. fetida without antibiotic. E. fetida* gut samples were collected on various days (days 1, 2, 3, 4, 7, and 10) from the experimental setup (i), while PrCD samples were collected on the same days (days 1, 2, 3, 4, 7, and 10) from all four experimental setups. Luria Agar (LA) plates were used to count total cultivable heterotrophic microorganisms. LA plates supplemented with oxytetracycline (15 g/ml) were used to calculate the fraction of heterotrophic bacteria resistant to oxytetracycline. HiCrome™ Bacillus Agar, (BA) (Hi-Media, M1651) plates were used to enumerate total *Bacillus* count from the samples [20]. BA supplemented with oxytetracycline (15 g/ml) was employed to obtain a fraction of *Bacillus* population resistant to oxytetracycline.

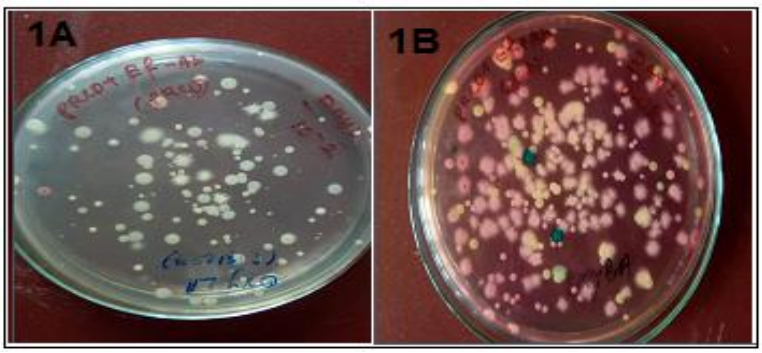

Fig. 1. Oxytetracycline supplemented Luria agar (1A) and Bacillus agar (1B) plates containing the resistant populations of cultivable heterotrophic bacteria and *Bacillus* spp. respectively from the gut content of the *E. fetida* reared in PrCD exposed to Sub-MIC level of oxytetracycline for 2 days. [PrCD-processed cow dung].

PrCD was weighed and placed in microfuge tubes before being diluted with PBS solution from each experimental set-up. Then after vortexing, it was serially diluted and spread plated in LA, BA, LA with oxytetracycline, and BA with oxytetracycline. Gut was dissected and homogenised in PBS solution to get gut samples from *E. fetida*. To dissolve the intestinal material properly, the homogenized solution was vortexed. The spread plating was then done in all four types of plates using varying dilutions. After that, the plates were incubated at 28 ^{0}C. On LA and oxytetracycline-amended LA (Fig. 1A), sensitive and oxytetracycline-resistant heterotrophic bacterial colonies were counted, while sensitive and oxytetracycline-resistant *Bacillus* colonies were counted on BA and oxytetracycline-amended BA, respectively (Fig. 1B). In each case, the plates were incubated overnight to determine the colony forming unit (cfu) value. The fraction of oxytetracycline-resistant cultivable heterotrophic bacteria and the resistant *Bacillus* population abundance in PrCD as well as in the gut of *E. fetida* were calculated using the cfu data obtained from both the LA and BA plates with or without oxytetracycline. The frequency of the mutation was determined using a conventional formula.

3. RESULTS AND DISCUSSION

There has been a significant log-fold increase [10.556 (on day 1) to 14 (on day 10); and from 8.519 (on day 1) to 12.88 (on day 10)] in the total heterotrophic bacterial count of PrCD samples left without adding earthworms and oxytetracycline and PrCD samples supplemented with subinhibitory concentration of tetracycline and left with earthworms respectively. This data reveals that the addition of subinhibitory concentration of oxytetracycline do not influence the normal growth of heterotrophic bacteria irrespective of the presence of *E. fetida* in the PrCD sample. On the other hand, the log cfu number of oxytetracycline-resistant heterotrophic bacteria in the population remained relatively constant from day 1 to day 10, with the exception of a log-fold increase (from 5 to 7.362) in oxytetracycline-supplemented PrCD samples left without earthworms, which could indicate that earthworms were not feeding live bacteria. As a result of the establishment of spontaneous mutants within the mixed bacterial community, there is a stable sub-population of oxytetracycline-resistant heterotrophic bacteria in PrCD [21-22]. The impact of antibiotics on the insect (an invertebrate) gut microbiome was previously investigated by discovering tetracycline resistance genes in the gut bacteria of *Galleria mellonella* larvae fed on oxytetracycline-containing non-natural food. *G. mellonella* was reared on non-natural food for more than five generations, and the larvae were found to tolerate low dosages of antibiotics in their diets, although oxytetracycline levels were greater than subinhibitory cause early larval mortality [23]

HiCrome™ Bacillus Agar used to study the trend in increase of *Bacillus* population is based on the formulation of MYP Agar formulated by Mossel et al (1967) used for enumeration of *Bacillus cereus* and *Bacillus thuringiensis* present in certain foods. These bacteria carry the enzyme beta-glucosidase that cleaves the chromogenic mixture present in the medium to produce blue colonies. Mannitol, present in the media is fermented by organisms like *B. megaterium* to produce yellow coloured colonies under presence of phenol red as indicator. The medium contains peptone which provides nitrogenous, carbonaceous compounds and other essential growth nutrients. HiCrome™ Bacillus Agar revealed the trend in increase of *Bacillus* population in all the four experimental PrCD samples which was evident from day 1 to day 10 of the experiment (Fig. 2). Similar was the trend noted for log cfu number of oxytetracycline-resistant *Bacillus* spp. in the population as it remained relatively constant from day 1 to day 10 (Fig. 2). In an earlier study of vertebrate animals, veal calves were given oxytetracycline at either a high therapeutic dose or a low therapeutic dose to mimic exposure to environmental contamination in an intervention research. Sub-therapeutic oxytetracycline dosing did not result in elevated *tet*M resistance levels as found in the therapeutic group under the conditions examined [24].

In the gut of *E. fetida,* fed with PrCD supplemented with or without subinhibitory concentration of oxytetracycline, a significant increase in total heterotrophic bacterial load was observed with the passage of time (Fig. 3). On the contrary, the sub-population of oxytetracycline-resistant heterotrophic bacteria remained fairly constant (log cfu value being 2.815 on day 1 and 2.322 on day 10 of the experiment) in the gut of *E. fetida* fed on PrCD supplemented with subinhibitory concentration of oxytetracycline, but the oxytetracycline-resistant heterotrophic bacterial population in the gut microbiota dropped at least by one order (log cfu

value being 3.477 on day 1 and 2.778 on day 10 of the experiment) when *E. fetida* was reared on PrCD without supplementation of oxytetracycline. The absence of antibiotic-selective pressure could be responsible for the partial depletion of the population of spontaneous mutants being outcompeted by the majority of wild-type population sensitive to oxytetracycline. The frequency of oxytetracycline-resistant *Bacillus* spp. (5×10^{-8}) on day 1 matched the frequency of spontaneous mutations, while higher frequencies on days 2 and 7 (1.6×10^{-4} and 3.5×10^{-6} respectively) revealed widespread oxytetracycline resistance in the gut environment. Resistance development was seen in the heterotrophic bacterial population (on LA plate) and the *Bacillus* population (on BA plate), similar to earlier research [25]. It was clear that antibiotics at subinhibitory doses have a significant impact on mutation rates, horizontal gene transfer, and biofilm formation, all of which could contribute to the emergence and spread of antibiotic resistance [26]. Bacteria acquire antibiotic resistance genes through three different mechanisms: conjugative transfer of resistance plasmids [27-28], transformation [29], and spontaneous mutation [30]. Several studies have found that antibiotics at subinhibitory concentrations (much below the MIC) can cause bacterial mutation rates to increase. Antibiotics could enhance antibiotic resistant microorganisms even at concentrations hundreds of times lower than the lowest inhibitory concentration [31].The molecular processes and evolutionary factors determining bacterial responses to subinhibitory antibiotic concentrations, therefore, should be thoroughly investigated.

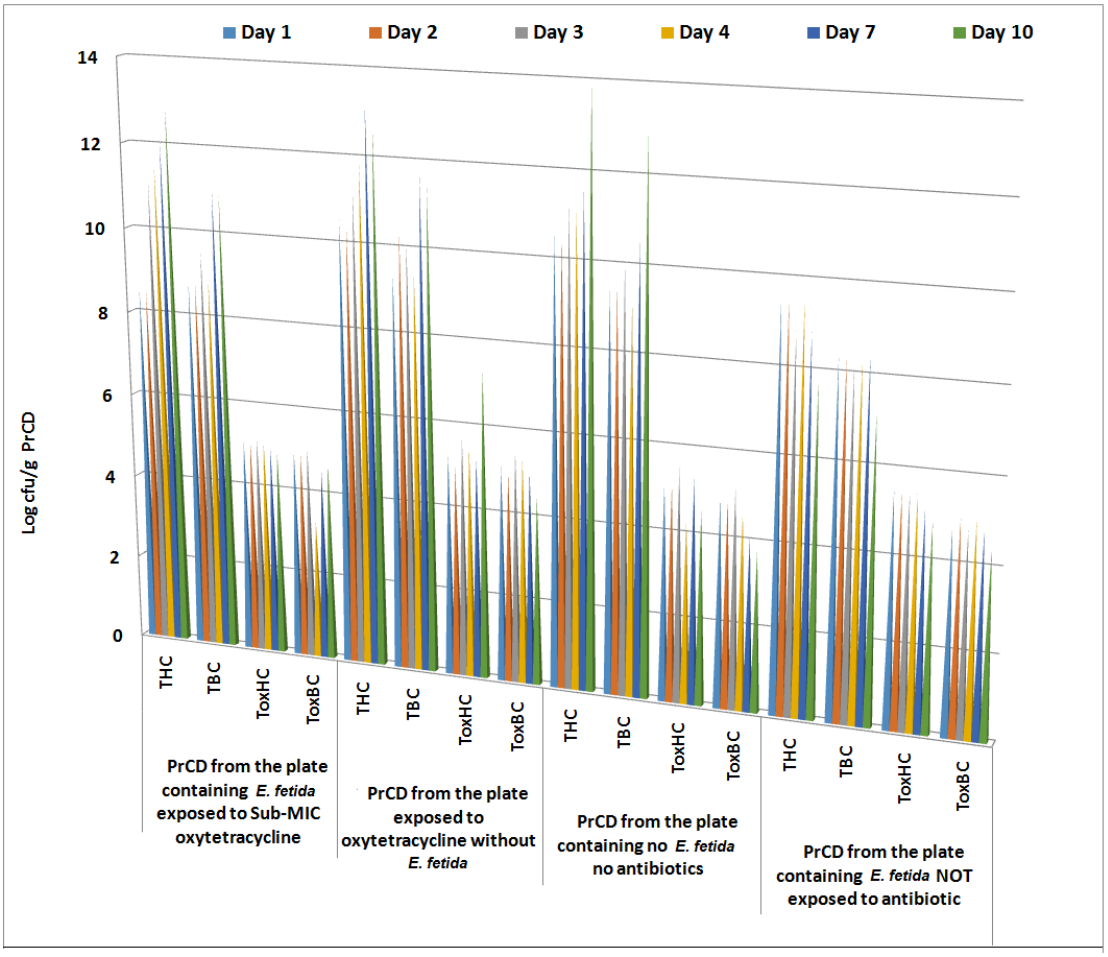

Fig. 2. Graphical representation of Bacterial population (mean Log cfu/g) in PrCD (shown in Y axis) from different experimental plates (shown in X axis) showing Total Heterotrophic bacterial count (THC), Total *Bacillus* spp. Count (TBC), Total oxytetracycline resistant heterotrophic bacterial count (ToxHC) and Total oxytetracycline resistant *Bacillus* spp. Count resistant (ToxBC). [PrCD-processed cow dung; Ef-*Eisenia fetida*]

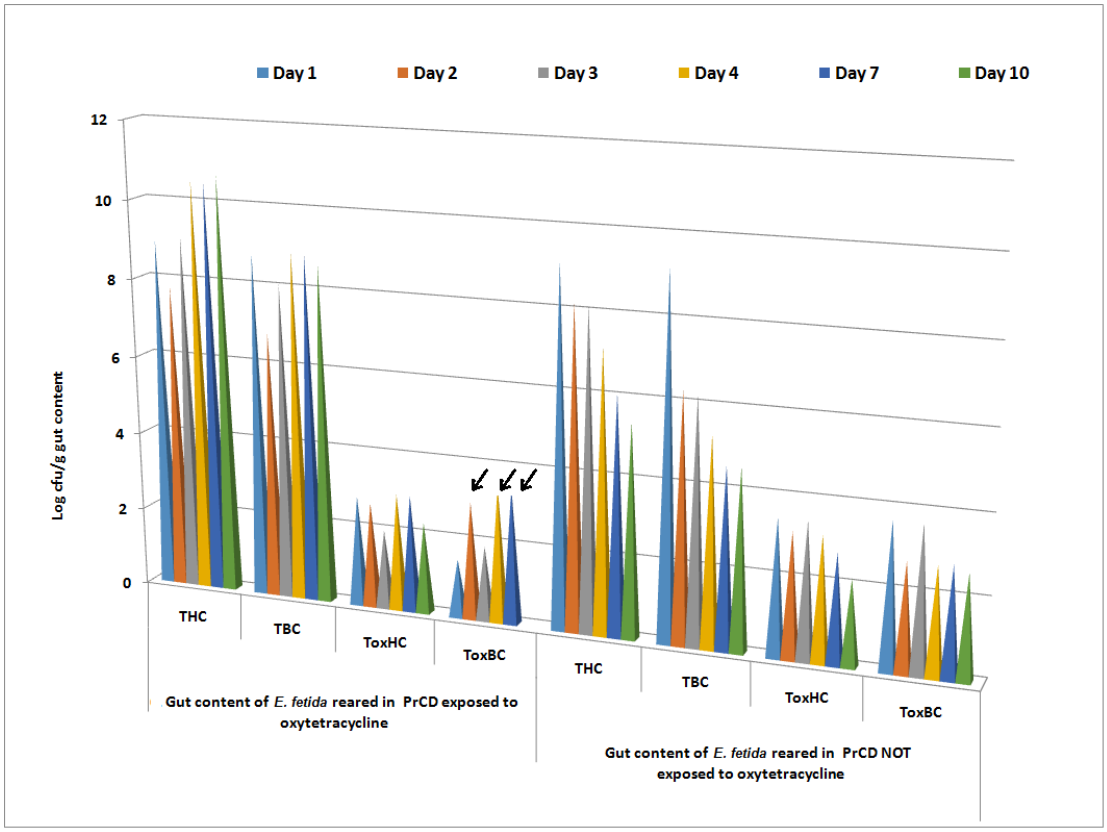

Fig. 3. Graphical representation of mean bacterial population (in cfu/g) of gut content of *E. fetida* (shown in Y axis) from different experimental plates (shown in X axis) showing Total Heterotrophic bacterial count (THC), Total *Bacillus* spp. Count (TBC), Total oxytetracycline resistant heterotrophic bacterial count (ToxHC) and Total oxytetracycline *Bacillus* spp. Count resistant (ToxBC). Arrow signs indicate significant increase of resistant *Bacillus* spp. in the gut of *E. fetida* [PrCD-processed cow dung; Ef-*Eisenia fetida*].

4. CONCLUSIONS

Bacteria resistant to oxytetracycline were isolated and counted in this study to evaluate how frequently they mutated and how susceptible they were to oxytetracycline stress. Subinhibitory oxytetracycline concentration in the earthworm diet was found to have a significant effect on *Bacillus* spp. mutation rates during passage through the gut of *E. fetida*, implying that evolutionary forces on the gut microbiota may be determining their responses to subinhibitory antibiotic dosages.

DISCLAIMER

The products used for this research are commonly and predominantly used products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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