Mineralization, Biodegradation, and Antagonistic Activities of Gut-associated Bacteria and Fungi of African Nightcrawler, *Eudrilus eugeniae* (Kinberg, 1867)

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ABSTRACT

Earthworms and their interactions with microorganisms offer beneficial effects that can improve organic matter decomposition, enhance nutrient availability, and suppress pathogens in the soil. In this study, microorganisms from the gut of *Eudrilus eugeniae* (Kinberg, 1867), commonly known as African nightcrawler or ANC, were isolated through pour plate method and screened for their activities using assays to confirm nitrogen fixation, phosphate solubilization, polyethylene utilization, and antagonistic potential. The identifications of eight bacterial and six fungal isolates were confirmed based on nearest phylogenetic affiliations. Fungal isolates *Aspergillus aculeatus*, *Aspergillus japonicus*, *Fomitopsis* sp., and *Penicillium citrinum* exhibited antagonistic activity against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Bacterial isolates *Aeromonas caviae* and *Bacillus xiamenensis* utilized low- and high-density polyethylene as carbon sources. These isolates were also found to have high phosphate solubilization index (2.55-2.67) with high amount of phosphate solubilized (*A. caviae*: 0.799; *B. xiamenensis*: 0.778) at decreasing pH (i.e. pH 7.0 to 4.0). *A. caviae* and *B. xiamenensis* also showed nitrogen-fixing activity which is supported by the detection of *nif*H gene (>300 bp) and high nitrogen content (50 kg/ha NO_3-N) of vermicasts. The activities of these gut-associated bacteria and fungi must be further explored to optimize the use of ANC's casts and compost for agricultural, medical, and other applications.

Keywords: antagonistic activity, earthworm, microorganisms, nitrogen fixation, phosphate solubilization, polyethylene utilization

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INTRODUCTION

Earthworms are considered "ecosystem engineers" owing to their role in nutrient cycling through vermicomposting (Lavelle and Martin 1992; Chapuis-Lardy et al. 1998). Vermicomposting facilitates degradation of a wide variety of materials and produces products for agricultural applications. For example, compost products of earthworm species *Eisenia fetida* (Savigny, 1826), *Eudrilus eugeniae* (Kinberg, 1867), *Lampito mauritii* Kinberg, 1867, *Perionyx ceylanensis* Michaelsen, 1904, and *Perionyx excavatus* Perrier, 1872 were proven to improve the growth and yield of bell pepper, cucumber, marigold, strawberry, tomato, and ornamental plants (Atiyeh et al. 2000; Azarmi et al. 2008; Singh et al. 2008; Karmegam and Daniel 2009; Zhao et al. 2017; Rekha et al. 2018).

The contribution of microbial interactions of earthworms in nutrient cycling through mineralization and organic matter decomposition has been reported (Lavelle and Martin 1992; Chapuis-Lardy et al. 1998; Bohlen et al. 2004; Aira et al. 2009). The presence of *Acinetobacter* spp., *Azotobacter* spp., *Bacillus* spp., *Clostridium* spp., *Halobacterium* spp., *Micrococcus lylae*, *Pseudomonas aeruginosa*, *Spirocheata* spp., *Staphylococcus aureus*, and *Streptococcus mutans* in the gut and casts of *Libyodrilus violaceus* Beddard, 1891 was associated with high rate of organic matter decomposition (Idowu et al. 2006). Bacteria capable of phosphate solubilization were detected in the gut of *Allolobophora chlorotica*, *Aporrectodea longa*, and *E. fetida* (Maheswari and Sudha 2013). *Acinetobacter baumanni*, *Lactobacillus pantheries*, *Virigibacillius chiquenigi*, and several species of *Bacillus* were isolated from epigeic *E. fetida* that was proven to efficiently degrade and convert paper cups into vermicompost (Arumugam et al. 2014). Paper, garden, and kitchen wastes were also degraded through the action of *E. fetida* (Wani et al. 2013; Amita and Joseph 2017).

The antagonistic potential of microorganisms associated with earthworms was also studied. The casts of *Pheretima posthuma* were found to harbor actinomycetes with antagonistic activity against human bacterial pathogens including *B. subtilis*, *Escherichia coli*, *P. aeruginosa*, and *S. aureus* (Kumar et al. 2012). Other studies on antagonistic activity involved testing extracts of earthworms against bacteria. The growth of *E. coli*, *Klebsiella pneumoniae*, *Staphylococcus epidermidis*, *Proteus vulgaris*, *P. mirabilis*, *P. aeruginosa*, and *S. aureus* was inhibited by *L. mauritii* and *P. excavatus* powder (Prakash and Gunasekaran 2011). The antagonistic activity of *L. mauritii* extracts against *Aeromonas hydrophila*, *B. subtilis*, *Salmonella typhi*, *S. aureus*, and *Vibrio parahaemolyticus* was also confirmed (Bhorgin and Uma 2014; Kathireswari et al. 2014). The extracts of *Lumbricus rubellus* Hoffmeister, 1843

were effective against the human pathogen *Porphyromonas gingivalis* (Dharmawati et al. 2019), while *Wegeneriona* sp. extracts were effective against *Serratia marcescens* (Dhanam et al. 2016). The extracts of *P. excavatus* and *P. posthuma* showed inhibitory activity against fish pathogens including *A. hydrophila*, *Enterobacter aerogenes, E. coli*, *Micrococcus luteus*, *P. aeruginosa*, *Pseudomonas fluorescens*, and *S. aureus* (Bansal et al. 2015).

Diverse bacteria and fungi were found to inhabit the gut of ANC (Bamidele et al. 2014) and other earthworm species such as *Aporrectodea caliginosa* (Savigny, 1826), *Eisenia andrei* Bouché, 1972, *E. fetida, L. violaceus, Lumbricus terrestris* Linnaeus, 1758, and *P. excavatus* (Toyota and Kimura 2000; Pižl and Nováková 2003; Idowu et al. 2006; Chowdhury et al. 2007; Byzov et al. 2009; Owa et al. 2013; Bamidele et al. 2014). The gut environment, characterized by different pH levels, moisture content, oxygen concentrations, and nutrient levels, affects the composition and metabolic activities of gut-associated microorganisms (Karsten and Drake 1995; Horn et al. 2003; Idowu et al. 2006). In turn, these microorganisms contribute to primary production, microclimate regulation, pollution remediation, and nutrient cycling of earthworms in the soil environment (Blouin et al. 2013).

ANC is an epigeic species that is commonly used in vermicomposting. The species is popular due to its fast growth (40-49 days to reach sexual maturity), voracious feeding, consumption of high volume of wastes, rapid decomposition of organic matter, and tolerance to adverse environmental conditions (Viljoen and Reinecke 1989; Reinecke et al. 1992; Dominguez et al. 2001; Monebi and Ugwumba 2013). In the Philippines, ANC was first introduced in the 1980s, and is currently being promoted by the Department of Agriculture for vermicomposting as the species prefers temperature ranging from 25 °C to 30 °C that is common in the tropics (Dominguez et al. 2001; Blakemore 2015). Composts processed by ANC are being used as fertilizer, such as for lowland and upland rice (Guerrero and Guerrero 2014; Blakemore 2015) and as feed, such as for Nile tilapia (*Oreochromis niloticus*) (Guerrero and Guerrero 2014).

Despite the prevalence and high rate of utilization of ANC for vermicomposting, there is generally lack of information on the composition, diversity, and activities of microorganisms associated with this species in the Philippines. This study aimed to isolate bacteria and fungi from the gut of ANC, and screen these microorganisms for nitrogen fixation, phosphate solubilization, polyethylene utilization, and antagonistic potential. The findings of this study may contribute to better understanding of the utilization of ANC for vermicomposting in relation to the beneficial activities of their gut-associated bacteria and fungi.

MATERIALS AND METHODS

Collection of Earthworm Samples

Earthworms, identified as *E. eugeniae* (African nightcrawler) following the description of Blakemore (2015) (Nonillon Aspe, *personal communication*), were collected along with their soil substrate from the vermicompost facility of Task Force Solid Waste Management (TFSWM), University of the Philippines Diliman (UPD), Quezon City, Philippines. Twenty adult earthworms, characterized by the presence of clitellum, were individually handpicked and placed in a container (17.3 cm x 11.8 cm x 3.8 cm) made of polypropylene (Owa et al. 2013). Soil samples were collected from the uppermost 10-40 cm of the vermicompost bed (Horn et al. 2003; Idowu et al. 2006). Samples were immediately transported to the laboratory for processing.

Earthworms were stored in a container provided with aeration and moist sterile filter paper. Fresh vermicasts were collected from the containers after 12-15 hours (Bityutskii and Kaidun 2008). Earthworms were then starved for 48-72 hours, surface sterilized with 70% ethanol for 30 seconds, washed three times with sterile distilled water, and kept frozen for 3-4 hours at –16 °C (Horn et al. 2003; Byzov et al. 2009; Mudziwapasi et al. 2016). Gut contents were obtained through dissection following the protocol of Owa et al. (2013).

Isolation and Purification of Bacteria and Fungi from ANC Gut

Bacteria and fungi were isolated from the gut samples through pour plate method (Byzov et al. 2009; Mudziwapasi et al. 2016). Briefly, 0.5 g of gut contents was suspended in 2.5 mL sterile distilled water (1:5 ratio) and vortexed until homogenized. Serial dilutions up to 10^{-7} were performed by adding 1 mL of the homogenized sample into 9 mL sterile distilled water. From the last two dilutions (10^{-6} and 10^{-7}), 1 mL aliquot was inoculated onto Nutrient Agar (NA) supplemented with nystatin for bacterial isolation and Potato Dextrose Agar (PDA) supplemented with chloramphenicol for fungal isolation. NA plates were incubated for 18-72 hours at 37 °C and for 7 days in anaerobic condition at room temperature while PDA plates were incubated for 5 days at 25-27 °C. For purification, colonies with distinct morphologies were selected and repeatedly sub-cultured (Idowu et al. 2006; Byzov et al. 2009; Owa et al. 2013; Bamidele et al. 2014).

Extraction of Bacterial and Fungal DNA

Pure bacterial isolates were subjected to DNA extraction using boil lysis method (Dashti et al. 2009; Barbosa et al. 2016). One mL of overnight culture of bacteria in Nutrient Broth (NB) were centrifuged and re-suspended in 100 µL sterile distilled water in a 1.5 mL sterile tube, vortexed for 15 seconds, and centrifuged at 13,100 rpm for 5 minutes. The supernatant was discarded and 100 µL sterile distilled water was added followed by centrifugation for 10 minutes. Pellets re-suspended in 5 µL sterile distilled water were boiled at 100 °C in a dry bath for 15 minutes and then centrifuged for 2 minutes. Supernatant containing the DNA was transferred into a new sterile tube.

Fungal DNA extraction was carried out following the protocol of Liu et al. (2000). A lump of mycelia grown in PDA was inoculated onto a sterile 1.5 mL tube with 500 µL lysis buffer and then left at room temperature for 10 minutes. After adding 150 µL potassium acetate solution, the tube was then vortexed, and centrifuged at 13,200 rpm for 1 minute. Supernatant was transferred into a new tube with equal volume of isopropyl alcohol, mixed, and centrifuged for 2 minutes. Pellets were washed with 300 µL 70% ethanol, and centrifuged at 10,000 rpm for 1 minute. After air-drying, pellets were dissolved in 50 µL 1x Tris-EDTA.

Molecular Identification of Isolated Bacteria and Fungi

Polymerase chain reaction (PCR) was performed using the universal primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1392r (5'-ACGGGCGGTGTGTRC-3') for bacteria (Furlong et al. 2002) and ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') for fungi (Martin and Rygiewicz 2005). For bacteria, the reaction mixture (25 µL) consisted of 12.5 µL of 2X *Taq* master mix, 1 µL of each primer, 2 µL bacterial DNA (control excluded DNA), and nuclease-free water. For amplification of 27f and 1392r (>500 bp), the PCR conditions were: initial denaturation for 2 minutes at 94 °C followed by 25 cycles of denaturation for 30 seconds at 94 °C, annealing for 30 seconds at 60 °C, extension for 45 seconds at 72 °C, and final extension for 7 minutes at 72 °C. For fungi, the reaction mixture (25 µL) consisted of 12.5 µL of 2X *Taq* master mix, 1 µL of each primer, 3 µL fungal DNA (control excluded DNA), and nuclease-free water. For amplification of ITS1 and ITS4 (>500 bp), the PCR conditions were: initial denaturation for 5 minutes at 95 °C followed by 35 cycles of denaturation for 30 seconds at 95 °C, annealing for 1 minute at 55 °C, extension for 1 minute at 72 °C, and final extension for 6 minutes at 72 °C. PCR products were electrophoresed on 1.5% agarose gel with GelRed in TAE buffer for 30 minutes at 80 V using a 100-bp molecular weight DNA marker and

then submitted to Macrogen (Korea) for purification and sequencing. The Basic Local Alignment Search Tool (BLAST) was used for sequence identification. Sequences were aligned and trimmed using BioEdit prior to construction of Bayesian inference (BI) tree using MrBayes version 3.1.2 and TreeView version 1.6.6.

In vitro Screening of ANC Gut-associated Fungi for Antagonistic Activity

The antagonistic activity of isolated fungi against *B. subtilis*, *E. coli*, *P. aeruginosa*, and *S. aureus* was evaluated using the antagonism test following the protocol of Suárez-Estrella et al. (2007). Briefly, a block from a 5d-old culture of fungi in PDA was placed at the center of Mueller-Hinton Agar (MHA) plate. Then, 100 µL of test organisms cultured in Nutrient Broth were spot inoculated approximately 2.5 cm from the block. Plates were incubated for at least 48 hours at 30-37 °C and checked for inhibition indicated by the absence of any contact between fungal isolates and test organisms.

In vitro Screening of ANC Gut-associated Bacteria for Polyethylene Utilization

Screening for polyethylene utilization was done by inoculating 100 µL of 18-24 h-old bacterial cultures from Nutrient Broth into 10 mL sterile Bushnell Haas (BH) broth supplemented with 0.3% low-density polyethylene (LDPE) and high-density polyethylene (HDPE) powder. All broth tubes were incubated for 7 days in a shaker at 37 °C with 180 rpm agitation and observed daily for turbidity to confirm the growth of bacteria that were able to utilize polyethylene as carbon source for bacterial growth.

In vitro Screening of ANC Gut-associated Bacteria for Nutrient Mineralization Activity

Ten µL of 18-24 h-old bacterial cultures from Nutrient Broth were spot inoculated onto Nitrogen-free Malate Media supplemented with bromothymol blue (BTB), Pikovskaya's Agar (HiMedia M520), and Aleksandrow Agar (HiMedia M1996), and incubated for 5 days at 37 °C. Cultures were observed every 24 hours for nitrogen fixation activity indicated by a blue coloration zone (Gothwal et al. 2008). Phosphorus and potassium solubilizations were indicated by a clearing zone. Phosphorus solubilization index (SI) was calculated based on colony and zone diameters (Shanware et al. 2014; Sharon et al. 2016).

Isolates positive for nitrogen fixation were subsequently subjected to molecular detection of *nif*H gene (>300 bp) (Szymanska et al. 2016a). PCR amplification was carried out using the *nif*H gene primers 19F (5'-GCXWTYTAYGGXAARGGXGG-3') and 388R (5'-AAXCCRCCRCAXACXACRTC-3'). The reaction mixture (23.5 µL) consisted of 10 µL of 2X *Taq* master mix, 0.125 µL of each primer, 13 µL of nuclease-free water, and 0.375 µL bacterial DNA (control excluded DNA). The PCR conditions were: initial denaturation for 5 minutes at 94 °C followed by 40 cycles of denaturation for 30 seconds at 94 °C, annealing for 1 minute at 50 °C, extension for 1 minute at 72 °C, and final extension for 5 minutes at 72 °C (Szymanska et al. 2016b). PCR products were electrophoresed on 1.5% agarose gel with GelRed in TAE buffer for 30 minutes at 80 V using a 100-bp molecular weight DNA marker.

Isolates positive for phosphate solubilization were further subjected to Murphy and Riley (1962) method for phosphate quantification. One µL of fresh bacterial culture in NB was inoculated into 50 mL of NBRIP (National Botanical Research Institute's Phosphate) medium at pH 7.0 supplemented with calcium phosphate ($Ca_3(PO_4)_2$) as sole source of phosphorus while medium without inoculum served as the control. All flasks were incubated for 72 hours at 24 ºC under constant agitation at 120 rpm (Matos et al. 2017). After centrifugation and filtration, the pH of the filtrate was measured using pH paper while phosphate content based on absorbance values was measured using spectrophotometer at 880 nm (Watanabe and Olsen 1965).

Measurement of Macronutrients in Soil and Vermicasts

Twenty grams each of soil and vermicast samples were air-dried overnight and sieved prior to analysis (Zhang and Schrader 1993; Aira et al. 2003; Hmar and Ramanujam 2014). The nitrogen (N), phosphorus (P), and potassium (K) content of these samples were measured using NPK soil test kit (HiMedia K054M) following manufacturer's instructions.

RESULTS AND DISCUSSION

ANC Gut-associated Bacteria and Fungi

A total of eight bacteria and six fungi were isolated from the gut of ANC (Table 1). The sequences of bacterial isolates showed highest similarities to *Aeromonas caviae* (99.37%), *Bacillus xiamenensis* (99.86%), *Bacillus thuringiensis* (98.54% - 99.86%), and *Paenibacillus xylanilyticus* (98.24%) (Figure 1). The sequences of fungal isolates showed highest similarities to *Aspergillus aculeatus* (99.12% - 99.82%), *Aspergillus japonicus* (99.17%), *Fomitopsis* sp. (99.23% - 99.83%), and *Penicillium citrinum* (99.02%) (Figure 2).

Table 1. Bacteria and fungi isolated from the gut of African nightcrawler or ANC, *Eudrilus eugeniae* (Kinberg, 1867)

	Nearest Phylogenetic Affiliation	Accession Number		Nearest Phylogenetic Affiliation	Accession Number
1	*Aeromonas caviae*	MG737573	1	*Aspergillus aculeatus*	JX291165
2	*Bacillus xiamenensis*	NR_148244	2	*Aspergillus aculeatus*	MH892845
3	*Bacillus thuringiensis*	JX994097	3	*Aspergillus japonicus*	KC128815
4	*Bacillus thuringiensis*	MN108016	4	*Fomitopsis* sp.	JQ067652
5	*Bacillus thuringiensis*	MG722793	5	*Fomitopsis* sp.	FJ372677
6	*Paenibacillus xylanilyticus*	KJ023382	6	*Penicillium citrinum*	MH427065
7	*Paenibacillus xylanilyticus*	JX281766			
8	*Paenibacillus xylanilyticus*	HF585011			

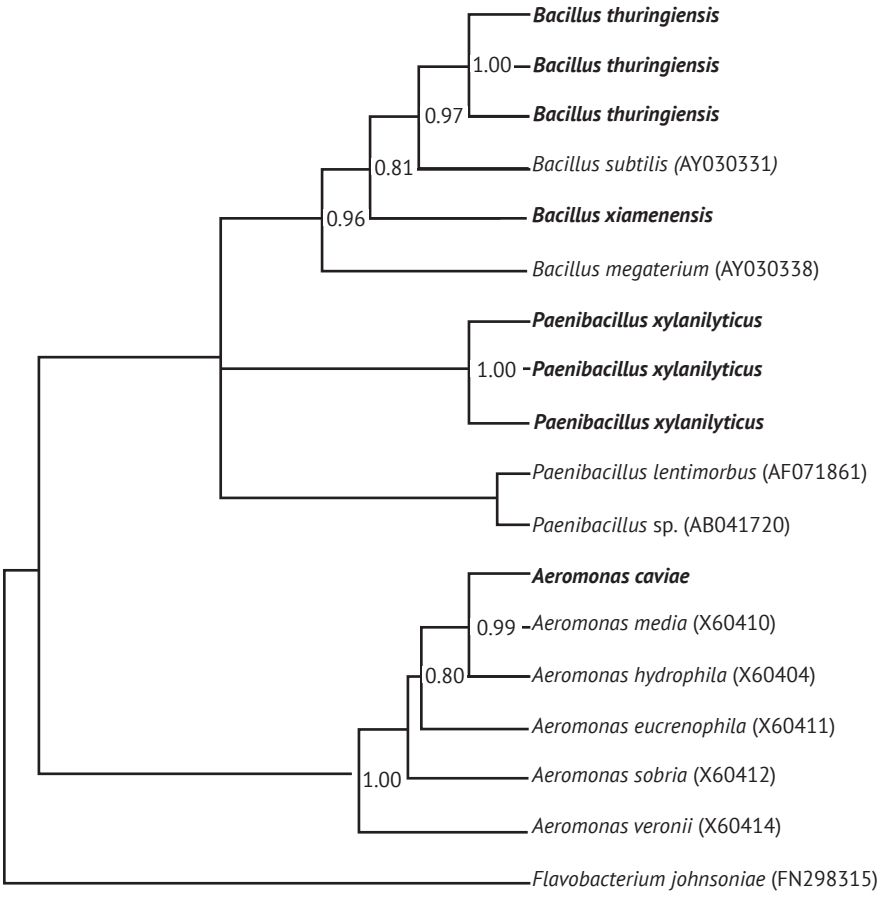

Figure 1. Bayesian inference tree of earthworm gut-associated bacteria based on 484 nucleotides. The tree is rooted on the Bacteroidetes *F. johnsoniae*. The number of generations and heating temperature used were 10,000,000 and 0.1, respectively. Numbers on nodes represent posterior probability values. Values less than 0.7 are not shown.

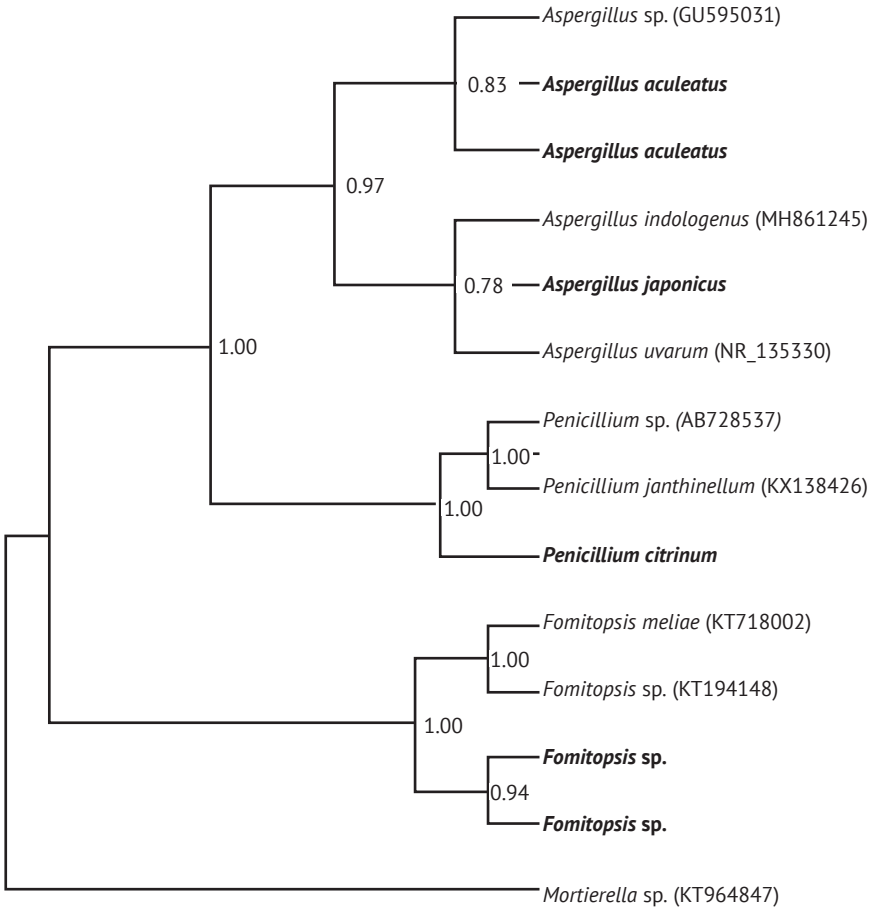

Figure 2. Bayesian inference tree of earthworm gut-associated fungi based on 587 nucleotides. The tree is rooted on the Mucoromycota *Mortierella* sp. The number of generations and heating temperature used were 10,000,000 and 0.125, respectively. Numbers on nodes represent posterior probability values. Values less than 0.7 are not shown.

B. xiamenensis was also isolated from the gut of ANC in India (Utekar and Deshmukh 2019). Other species isolated from the gut of ANC in India include *Bacillus pumilus* (Shankar et al. 2011), *B. aerius*, *B. licheniformis*, *B. safensis*, *B. subtilis*, *B. tropicus* (Utekar and Deshmukh 2019), *B. cereus*, and *B. subtilis* (Emperor and Kumar 2015; Govindarajan and Prabaharan 2015a, 2015b). Published studies on the isolation of *A. caviae* from earthworm gut are limited, but its occurrence in seafood, aquafarms, and mangroves (Joseph et al. 2013) as well as association with diarrhea/gastroenteritis (Dwivedi et al. 2008) were reported. The gut of ANC was also found to be inhabited by *Aspergillus flavus*, *A. fumigatus*, *A. nidulans*, *A. niger*, and *A. ochraceous* (Parthasarathi et al. 2007; Bamidele et al. 2014; Emperor and Kumar 2015) as well

as *Penicillium* sp. (Bamidele et al. 2014; Sahoo et al. 2015) while its vermicompost was found to have *P. citrinum* (Emperor and Kumar 2015).

Antagonistic Activity of ANC Gut-associated Fungi

The antagonistic activity of gut-associated fungi against Gram-positive *B. subtilis* and *S. aureus* and Gram-negative *E. coli* and *P. aeruginosa* was confirmed (Table 2). All fungal isolates showed activity against *B. subtilis*, *E. coli*, and *P. aeruginosa*. Growth of *S. aureus* was also inhibited by the fungal isolates except *A. aculeatus*. There is lack of information on the antagonistic activity of fungi associated with the gut of ANC, with most of the studies reporting the activity of its paste. ANC paste was reported to inhibit the growth of *B. subtilis, E. coli*, *K. pneumoniae,* and *S. aureus* (Vasanthi et al. 2013; Chauhan et al. 2014; Sethulakshmi et al. 2018).

Table 2. Antagonistic activity of fungi isolated from the gut of African nightcrawler or ANC, *Eudrilus eugeniae* (Kinberg, 1867)

Test Organisms	A. aculeatus	A. aculeatus	A. japonicus	*Fomitopsis* sp.	*Fomitopsis* sp.	P. citrinum
Bacillus subtilis	+	+	+	+	+	+
Escherichia coli	+	+	+	+	+	+
Pseudomonas aeruginosa	+	+	+	+	+	+
Staphylococcus aureus	-	-	+	+	+	+

(+) with antagonistic activity, (-) without antagonistic activity

The antagonistic activity of *Aspergillus*, *Fomitopsis*, and *Penicillium* isolated from organisms other than earthworms was also reported. *A. aculeatus* isolated from *Avicennia marina* (black mangrove along Red Sea) and *A. japonicus* isolated from *Tridax procumbens* (coat button or tridax daisy) inhibited the growth of *B. subtilis*, *E. coli*, *K. pneumoniae*, *P. vulgaris*, *Salmonella typhimurium*, *S. aureus*, and *Streptococcus pyogenes* (Aharwal et al. 2018; Basheer et al. 2018). The activity of *A. aculeatus* against Gram-positive and -negative bacteria was associated with its secondary metabolites namely ergosterol, ergosterol peroxide, secalonic acid D and F, variecolactone, and variecolin (Yodsing et al. 2017). *Fomitopsis feei*, *F. lilacinogilva*, and *F. rosea* collected from India, Australia, and Philippines respectively, were tested to be effective against the above mentioned bacteria as well as *E. aerogenes*, *M. luteus*, and *P. mirabilis* (Bala et al. 2011; Nidadavolu et al. 2011; Gaylan et al.

2018). *P. citrinum* isolated from marine and soil samples inhibited the growth of *B. subtilis*, *E. coli*, *S. typhi*, and *S. aureus* (Christophersen et al. 1998; Gharaei-Fathabad et al. 2014). The inhibition was attributed to the production of mycotoxin citrinin, which was also found to be effective against *B. cereus*, *B. pumilus*, *B. subtilis*, *E. coli*, *K. pneumoniae*, *Lactobacillus arabinosus*, *P. mirabilis*, *S. typhi*, *S. typhimurium*, *Shigella boydii*, *S. dysenteriae*, *S. sonnei, S. aureus*, *Streptococcus pneumoniae*, and *Vibrio cholerae* (Mazumder et al. 2002).

Polyethylene Utilization of ANC Gut-associated Bacteria

A. caviae and *B. xiamenensis* utilized both low-density polyethylene (LDPE) and high-density polyethylene (HDPE) after 120 hours of incubation (Figure 3). Members of *Bacillus* (*B. mycoides* and *B. subtilis*) isolated from mangrove soil were reported to degrade LDPE and HDPE (Ibiene et al. 2013) while *B. megaterium* isolated from plastic dumpsite soil was reported to degrade polyethylene in general (Mahalakshmi and Siddiq 2015). Other types of polymers such as polyethylene terephthalate (PET), polypropylene (PP), and polystyrene (PS) were degraded by *Bacillus* species from mangrove sediments and soil samples (Asmita et al. 2015; Auta et al. 2018). Reduction of polymer mass by 4% confirmed the utilization of PP by *Bacillus* sp. for growth after 40 days of incubation (Auta et al. 2018). Bioremediation of soil polluted with diesel was also associated with ANC action, along with the reduction of the concentrations of arsenic, cadmium, chromium, copper, lead, mercury, nickel, and vanadium (Ekperusi and Aigbodion 2015). Another earthworm species, *L. terrestris*, reduced 60% of LDPE particle size within four weeks through the action of bacteria (Firmicutes and Actinobacteria) associated with its gut (Lwanga et al. 2018).

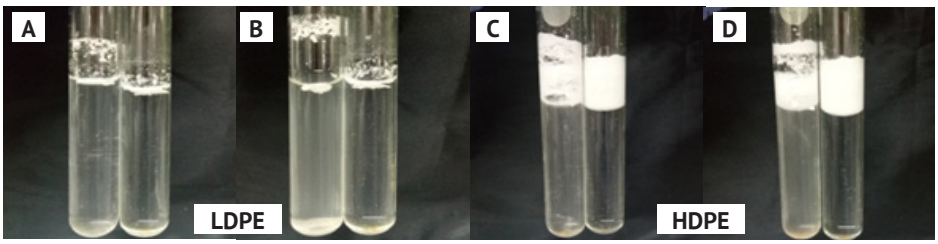

Figure 3. Low-density polyethylene (LDPE) and high-density polyethylene (HDPE) utilization by two bacteria, *Aeromonas caviae* (A, C) and *Bacillus xiamenensis* (B, D), shown by turbidity (left tube) compared with the negative control (right tube).

Nutrient Mineralization Activities of ANC Gut-associated Bacteria

A. caviae and *B. xiamenensis* were found to solubilize phosphate with high SI values of 2.67 and 2.55, respectively. After 22 hours of incubation, clearing zones were first

observed on Pikovskaya's agar medium, with maximum activity detected at 96 hours (Figure 4). The amounts of phosphate solubilized by *A. caviae* and *B. xiamenensis* were higher than the control (Figure 5) with observed decrease in pH (from 7.0 to 4.0). The two phosphate solubilizing isolates were also able to fix nitrogen on nitrogen-free malate medium as indicated by blue colored zones first observed after 96 hours of incubation, with maximum activity at 120 hours (Figure 4). The target *nif*H gene was detected in these isolates (Figure 6).

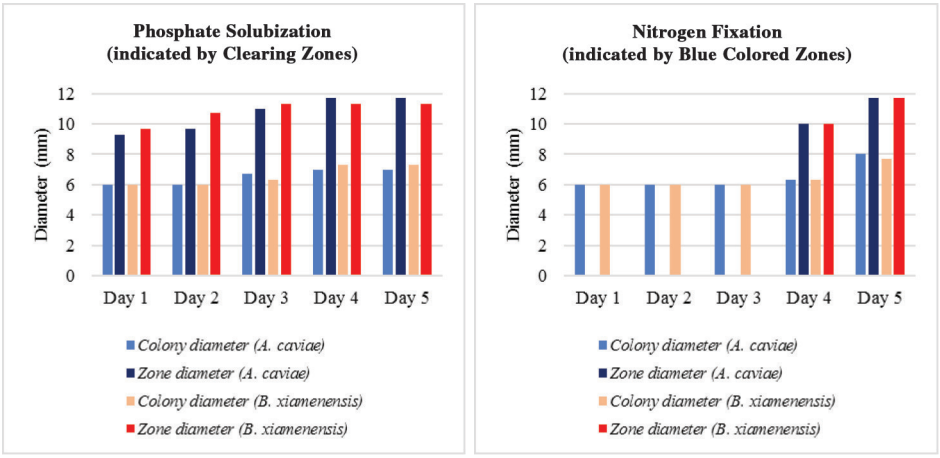

Figure 4. Diameter of clearing zones and blue colored zones produced by bacteria isolated from the gut of ANC (n=3). Colony diameter = diameter of bacterial growth; zone diameter = diameter of clearing/blue colored zone.

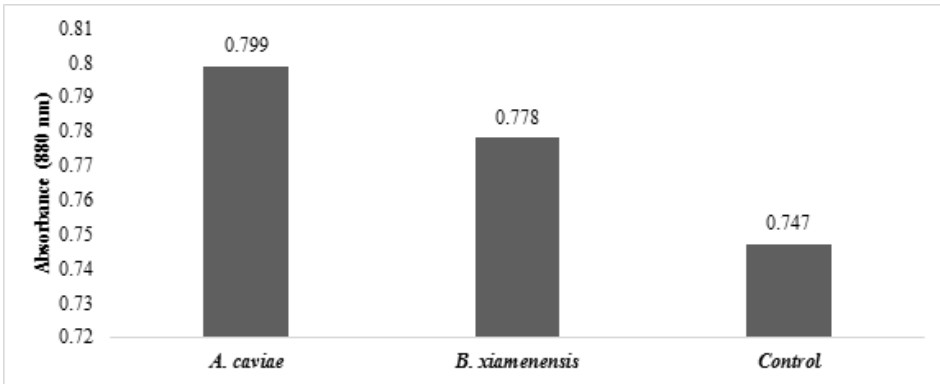

Figure 5. Amount of phosphate solubilized by two bacteria, *Aeromonas caviae* and *Bacillus xiamenensis*.

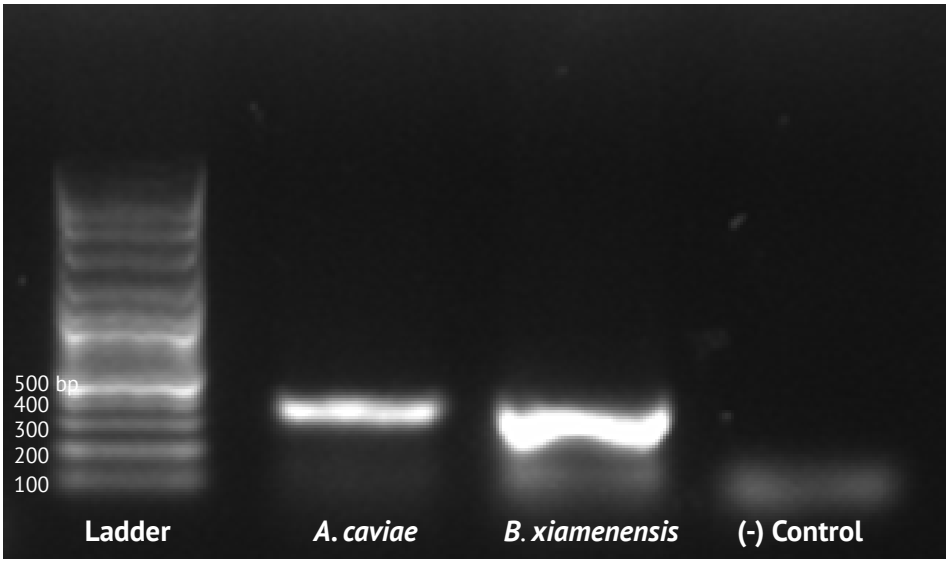

Figure 6. Molecular detection of *nif*H gene in two bacterial isolates, *Aeromonas caviae* and *Bacillus xiamenensis*.

The presence of phosphate solubilizing bacteria (PSB) and nitrogen-fixing bacteria (NFB) in the gut of ANC was previously reported in India (Albasha et al. 2014; Sequeira and Chandrashekar 2015; Khobragade and More 2016). The mechanisms involved in phosphate solubilization include ion-exchange, chelation, acidification, and organic acid production (Chen et al. 2006). In this study, the confirmed mineralization activities exhibited by the bacterial isolates might be associated with the high P and N concentrations in vermicasts (Lee 1992; Zhang et al. 2000; Shamini and Fauziah 2014; Prabha et al. 2015).

Bacteria capable of phosphate solubilization and nitrogen fixation were also isolated from the gut of other earthworm species. Epigeic *E. fetida* was found to be inhabited by gut-associated PSB and NFB (Hussain et al. 2016). The nitrogenase activity of earthworms in the gut of anecic *L. terrestris* as well as endogeic *Aporrectodea rosea* and *A. caliginosa* (Umarov et al. 2008) was evaluated. As it is in the present study, the maximum mineralization activity of bacteria associated with endogeic *Metaphire posthuma* was observed at 96 hours of incubation, the period at which bacteria might have reached exponential phase (Biswas et al. 2018). Likewise, *Aeromonas salmonicida* and *A. caviae* were reported elsewhere to show phosphate solubilization activity on Pikovskaya's medium after 5 days of incubation (Chen et al. 2012). *Aeromonas vaga* showed solubilization efficiency when subjected to varying temperatures (15, 25, 35, and 45 °C) and 8% sodium chloride (NaCl) at

pH 10 (Jha et al. 2013). *Aeromonas allosaccharophila*, *A. hydrophila*, and *A. media* isolated from rhizospheric soil were also confirmed to be PSB (Aarab et al. 2015).

The SI values for the phosphate solubilization activity of *A. caviae* and *B. xiamenensis* ranged from 2.55 to 2.67, which is comparatively higher than the reportedly high SI of 2.0 for *Aeromonas* sp. isolated from rhizospheric soils of cabbage fields in Iran (Motamedi et al. 2016). Species of *Bacillus* such as *B. cereus*, *B. megaterium*, *B. simplex*, and *B. subtilis* are also known phosphate solubilizers (Bahadir et al. 2018; Saeid et al. 2018; Zheng et al. 2018). *Bacillus* sp. and *B. pumilus* isolated from banana tree roots (Matos et al. 2017) as well as *B. subtilis* and *B. tequilensis* isolated from lentil rhizosphere in Ethiopia (Midekssa et al. 2015) solubilized calcium phosphate. *Bacillus* spp. capable of phosphate solubilization were isolated from ANC gut and vermicasts (Albasha et al. 2014). As what was observed in the present study, there was a decrease in pH with increased amount of P solubilized by rhizospheric *Aeromonas* (Kundu et al. 2009) and *Bacillus* species (Matos et al. 2017; Mohamed et al. 2018). The decrease in pH was asserted to be directly proportional to increased P solubilization due to acidification from secretion of organic acids (Mohamed et al. 2018).

Nitrogen-fixing *A. hydrophila* and *Aeromonas* sp. were isolated from rice fields (Xie et al. 2003) and from the rhizosphere of cabbage (Motamedi et al. 2016), respectively. The genus *Bacillus* is known to have nitrogen-fixing species namely *B. azotoformans*, *B. brevis*, *B. cereus*, *B. licheniformis*, *B. megaterium*, *B. pumilus*, and *B. subtilis* (Xie et al. 2003). *B. subtilis* isolated from the rhizosphere of ground nut exhibited nitrogen-fixing activity (Satapute et al. 2012). The detection of *nif*H in *Aeromonas* sp. (Flores-Mireles et al. 2007) and in *Bacillus alkalidiazotrophicus*, *B. arseniciselenatis* (Sorokin et al. 2008), and *B. cereus* (Emmyrafedziawati and Stella 2018) was done to support the findings on their nitrogen fixation activity. The gene has been the biomarker of choice for NFB as it encodes for the nitrogenase reductase subunit of nitrogenase enzyme involved in nitrogen fixation (Emmyrafedziawati and Stella 2018).

The amount of P was higher in vermicasts (56-73 kg/ha) than in the soil substrate (22-56 kg/ha). This is consistent with previous reports noting high P content of vermicomposts processed by ANC (Shamini and Fauziah 2014; Prabha et al. 2015) and in vermicasts of *Allolobophora caliginosa* (Sharpley and Syers 1976), *L. terrestris* (Le Bayon and Binet 2006), *Metaphire tschiliensis tschiliensis* (Teng et al. 2012), *Drawida* sp., *Eutyphoeus mizoramensis*, *Metaphire houlleti*, *P. excavatus*, and *P. macintoshi* (Hmar and Ramanujam 2014). The release of P in vermicasts is attributed to solubilization of microorganisms during gut passage (Lee 1992; Zhang et al. 2000).

Likewise, nitrogen content, measured as nitrate nitrogen (NO_3-N) in vermicasts, was found to be more than twice (50 kg/ha) that of the soil substrate (20 kg/ha). Several studies confirmed the same observation in the vermicasts of *Allolobophora molleri*, *A. caliginosa* (Aira et al. 2003), *L. violaceus* (Idowu et al. 2006), *L. terrestris*, *Octolasion cyaneum* (Buck et al. 1999), and *M. tschiliensis tschiliensis* (Teng et al. 2012) and in vermicomposts processed by ANC (Prabha et al. 2015), *E. fetida*, and *P. excavatus* (Mistry et al. 2015). Higher nitrogen content in vermicasts was associated with the activity of microorganisms that promote mineralization process (Mistry et al. 2015).

The degradation of organic wastes through vermicomposting is essential for nutrient cycling (Yi-Wei et al. 2012). Wastes reported to be efficiently degraded by ANC through vermicomposting include rice straw (Yi-Wei et al. 2012), coir pith (Nattudurai et al. 2014), and biogas plant slurry (BPS) (Rajeshkumar and Ravichandran 2015). Degradation of rice straw was completed by ANC in a shorter time (134 days) compared to *P. excavatus* (171 days), resulting to higher nutrient content in vermicasts (Yi-Wei et al. 2012). Moreover, it only takes 60 days for ANC to degrade coir pith, which usually takes longer time to degrade due to its lignin-cellulose complex (Nattudurai et al. 2014). Degradation of BPS was found to be enhanced by ANC as indicated by the decrease of total organic carbon (Rajeshkumar and Ravichandran 2015). Composts processed by ANC caused increase in plant height and weight, as well as increase in the length of shoots, roots, leaves, and root hairs of agricultural crops *Cyamopsis tetragonoloba* (cluster bean) (Nattudurai et al. 2014) and *Vigna radiata* (mung bean) (Rajeshkumar and Ravichandran 2015). Improved growth and yield of plants treated with vermicomposts were attributed to increased concentrations of NPK (Nattudurai et al. 2014; Rajeshkumar and Ravichandran 2015).

CONCLUSIONS

Eight bacteria and six fungi were isolated from the gut of ANC. The fungi identified as *A. aculeatus*, *A. japonicus*, *Fomitopsis* sp., and *P. citrinum* exhibited antagonistic activity against *B. subtilis, E. coli, P. aeruginosa,* and *S. aureus*. Among gut-associated bacteria identified as *A. caviae*, *B. xiamenensis*, *B. thuringiensis*, and *P. xylanilyticus*, the first two were found to utilize LDPE and HDPE as carbon sources for bacterial growth, indicating plastic biodegradation potential. Both isolates yielded high phosphate solubilization index and showed nitrogen fixation activity supported by the presence of *nif*H gene. High concentrations of nitrogen and phosphorus in the vermicasts of ANC may be associated with the confirmed mineralization activities.

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CONFLICTS OF INTEREST

MRFM and MCMO declare that they have no conflicts of interest.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

MRFM and MCMO conceived the study, designed the experiment, and analyzed data. MRFM performed most of the procedures. MCMO wrote the proposal and received the funding for the research. Both authors contributed to manuscript writing.

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