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Deciphering the Dietary Code: How Water Hyacinth to Secondary Sewage Sludge Ratios Orchestrate Earthworm Gut Microbiome Dynamics

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Abstract: Leveraging Water hyacinth (WH) and Secondary sewage sludge (SS) as a substrate for earthworms (*Eisenia fetida*) presents a burgeoning frontier within the realm of organic matter recycling and waste management. This study investigates the effect of feeding the same feed (WH + SS) in different proportions on the composition and diversity of earthworm gut microflora. In a controlled condition, earthworms were fed with two diets (Set 1: 75% SS + 25% WH; Set 2: 50% SS + 50% WH). After 45 days of feeding period, gut microflora analysis was carried out using high-throughput sequencing. The taxonomic profiling of earthworm gut showed significant alterations in bacterial composition. Despite the change, the gut bacterial community maintains a core microbiome that was present in both sets. Proteobacteria was identified as a predominant phylum. The abundance of Actinobacteria decreased with a higher amount of WH, while the abundance of Bacteroidetes increased with WH. The taxonomic to phenotypic analysis revealed that sulphur oxidizers and reducers were high in the gut with high amount of SS. Earthworms having diet with higher WH showed high abundance of cellulose and lignin degraders. Core microbiome is comprised of microbes associated with biogeochemical cycles, bioremediation, production of plant growth hormones, and phosphorus stabilization. The study illuminates the nuanced interplay between earthworms, their gut microflora, and substrate materials, delineating a framework for integrated waste valorisation strategies.

Keywords: *Eisenia fetida*, Bacterial succession, Sewage sludge, Water hyacinth, Metagenomics, Microbiome

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Introduction

Sewage sludge is a by-product of sewage treatment plants (STP) in form of solid, semi-solid, or slurry. During wastewater treatment, STP generates two types of sewage sludge that are Primary sewage sludge and Secondary sewage sludge. Wastewater treatment in a typical STP starts with preliminary treatment to remove coarse solid particles from wastewater. It continues with primary treatment in which some residues settle down when wastewater passes through primary classifiers. These residues are termed Primary Sewage Sludge. The next

treatment is secondary treatment for removal of biodegradable material using biological processes (use of microbes). This treatment leaves behind a concentrated suspension called Secondary Sewage Sludge (SS) or Biological sludge (Demirbas *et al*., 2017). SS composition normally depends on the origin of wastewater and the process by which it is separated. Common components of proteins, polysaccharides, and hydrogen and electrostatic bonds dominating microbes. These bonds are the reason for extracellular polymeric substance formation (Markis *et al*., 2016). It is also composed of polycyclic aromatic hydrocarbons (PAH), furans, dioxins, polychlorinated biphenyls (PCB), personal care products, pharmaceutical residues, heavy metals, detergent, endocrine disruptors, and synthetic steroids (Hudcová *et al*., 2019; Pola *et al*., 2021). Despite these toxic elements and pathogens, SS contains high amount of organic matter and nutrients that can improve soil quality and plant growth (Dume *et al*., 2022). Because of this, so many scientists successfully carried out vermin-composting of sewage sludge amended with some organic wastes to overcome its disposal problems.

Water Hyacinth (WH) is identified as one of the 100 most aggressive invasive species and one of the top 10 worst aquatic weeds by the IUCN (International Union for Conservation of Nature) (Téllez *et al*., 2008). It is resistant to chemical, mechanical, biological, and hybrid methods used for its removal. So, it is one of the hardiest weeds among all weeds. For removal of WH, its biomass can be used in production of Biogas, Biochar, Compost, Cattle, and fish feed, etc. It is mostly used for composting because of its decaying nature. The plant is also rich in phytochemicals proteins, and minerals. This property makes WH a good substrate for vermicomposting.

In this study both the waste substrates (SS and WH) were used for the earthworm (*Eisenia fetida*) feed formulation in order to vermicomposting. This research aims to investigate the impact of feeding WH and SS to earthworms on the composition and diversity of gut microflora using a metagenomic approach. Understanding the dynamics of gut microflora in response to these waste substrates can provide valuable insights into the role of earthworms in the conversion of SS spiked with WH to a nutrient-rich product. This is a follow up from our previous study where it was concluded that SS and WH can be converted into a good quality vermicompost using *Eisenia fetida* and procured vermicompost can be beneficial for plants.

Materials and Methods

Earthworm and Waste Collection

Fresh water hyacinth (*Pontederia crassipes*) (WH) was collected from different locations of Tapi River, Surat, Gujarat, India. The whole plant was washed and sundried for 15 days. Dried water hyacinth was powdered using a laboratory blender. Secondary sewage sludge (SS) was provided by Sewage Treatment Plant, Anjana, Surat, Gujarat, India. It was sundried for 7 days to form powder material. The powdered materials of both substrates were sieved with 0.5 mm sieve before being taken for preparation of feeding material.

Earthworms (*Eisenia fetida*) were collected from Ambikaniketan Gaushala, Kosmada, Surat, Gujarat, India. It was maintained on lignocellulolytic biomass feed (cow dung) at Department of Biosciences, Veer Narmad South Gujarat University, Surat, Gujarat, India. After 30 days, adult clitellated earthworms were used for experiment.

Feeding trials

A rectangular plastic container (130 cm × 65 cm × 50 cm) was used for experiment. Holes were made at the bottom of each container for drainage. The unit was set up in a greenhouse with temperature range from 28.4°C to 32°C. The powdered WH and SS were mixed at a ratio of 75% SS + 25% WH (Set 1) and 50% SS + 50% WH (Set 2) by weight. These two feed mixtures were then pre-composted for 7 days to remove volatile gases. 100 adult earthworms were fed with each feeding material for 45 days. Moisture level was maintained upto 70% by

spraying water. The experiment was performed in triplicates.

DNA extraction from intestinal content

Earthworms from each set were washed with tap water to remove feed particles. For sterilization of outer surface, 70% alcohol was taken. Again, earthworms were washed with sterile deionized water. The gut samples of the earthworm were acquired by squeezing anterior to posterior end aseptically (Biswas *et al*., 2018). Phosphate buffer was added to the gut sample (pH 8) and shaken with help of an orbital shaker at 150 rpm. The pellet was obtained by centrifugation. Same process was done to wash pellets. DNA extraction was carried out after this pretreatment of sample with modified method of previous studies (Zhou *et al*., 1996; Ruifu *et al*., 2003; Yang *et al*., 2007). DNA quality was checked on 0.8% agarose gel as well as BioTeK Epoch was used to determine A260/280 nm ratio. Qubit dsDNA HS Assay kit was used to quantify DNA. After this DNA concentration was determined using Qubit® 2.0 Fluorometer.

Metagenome Sequencing

DNA amplification and library preparation was done using Ion 16S Metagenomics Kit (Thermo Fisher Scientific, MA, USA) in combination with Ion Plus Fragment Library kit (Thermo Fisher Scientific, MA, USA) according to the manufacturer's workflow. The sequencing was carried out by Ion Torrent S5 Plus system (Thermo Fisher Scientific, MA, USA). The results were collected in FASTQ format.

Bioinformatic analysis

The raw sequence (FASTQ) was uploaded to One-Codex platform in order to carry out community structure analysis (Minot *et al*., 2015). With adapter trimming low quality and noisy portions were removed. For dereplication of redundant sequences, k-mer based approach was used. This quality control was followed by annotation process using BLAST-like alignment tool search (BLAT). Then the processed sequence was clustered with UCLUST at 97% sequence similarity threshold. The targeted Loci database from One-Codex platform was used to characterize the composition and diversity of microbial communities at different taxonomic levels. Diversity analysis was carried out with PAST (Paleontological Statistics Software Package for Education and Data Analysis) (Hammer *et al*., 2001). MEGAN (MEtaGenome Analyzer) was used to compare metagenomes and construct the core microbiome of earthworm gut (Huson *et al*., 2007). Taxonomic to phenotypic mapping of data was achieved by METAGENassist server (Arndt *et al*., 2012) by uploading taxonomic abundance data obtained from MEGAN. The same taxonomic abundance data was used to perform statistical analysis with STAMP (Statistical Analysis of Taxonomic and Functional Profiles) (Parks *et al*., 2014). Using Fisher's exact test, statistical differentiation of taxa abundance between groups was analyzed in STAMP. Taxa with <0.05 P-value were designated as significantly different in abundance between experimental groups.

Results

Growth Performance

The earthworm survival rate was 100% in both treatments. The experiment ended with final earthworm count of 112±2.0 and 108±1.84 in sets 1 and 2, respectively. The initial weight of earthworm was 2.53±1.05 g/earthworm, which was increased to 2.94±0.96 and 3.17±0.54 g/earthworm in sets 1 and 2 after experiment, respectively.

DNA Quality Control and Sequencing

0.8% agarose gel gave a clear observation of isolated DNA. The A260/280nm ratio confirms the purity of DNA, which was 1.836 and 1.812 for sets 1 and 2, respectively. Ion torrent sequencing yielded 1993772 total sequences with an average sequence length of 206±59 from earthworms of set 1 post-QC. It was 1804501 total sequences with an average sequence length of 201±45 for set 2. G+C content for each sequence was 55%.

Table 1: Alpha Diversity Matrices

Matrices	Set 1	Set 2
Taxa_S	1020	1522
Individuals	1122555	1322096
Dominance_D	0.01464	0.004029
Simpson_1-D	0.9854	0.996
Shannon_H	5.295	6.333
Evenness_$e^{H/S}$	0.1954	0.3697
Brillouin	5.292	6.328
Menhinick	0.9627	1.324
Margalef	73.15	107.9
Equitability_J	0.7643	0.8642
Fisher_alpha	110.6	169.9
Berger-Parker	0.05316	0.01785
Chao-1	1020	1522

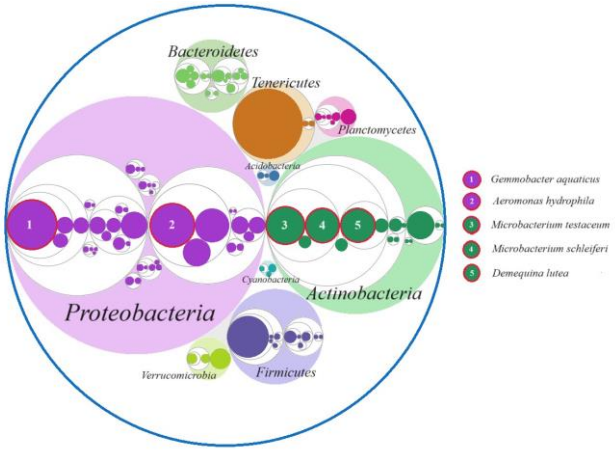
Table 2: Beta diversity

Whittaker	0.40519
Harrison	0.40519
Cody	515
Routledge	0.11345
Wilson-Shmida	0.40519
Mourelle	0.40519
Williams	0.14782

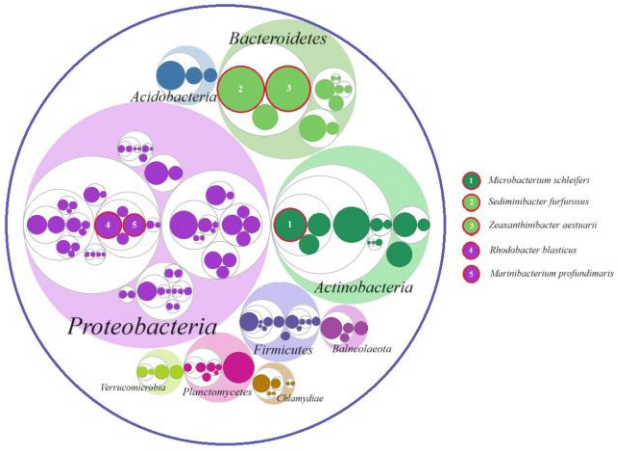
Taxonomic Profiling

Alpha diversity for each sample was calculated as the Shannon-Wiener index (H) and Simpson diversity index. Simpson diversity index (0.9) was quite similar for both samples, while Shannon-Wiener index was 5.295 and 6.333 for sets 1 and 2, respectively. Alpha diversity matrices other than these two are presented in Table 1. In case of Dominance index, Menhinik index, and Margalef index, adequate difference was observed between two samples. For set 1, Dominance index was quite higher (0.01464) compared to set 2 (0.004029). On the other hand, Menhinik index and Margalef index were higher in set 2 than in set 1. When it comes to beta diversity, Whittaker and Harrison beta diversity was 0.40519. Cody, Routledge, Wilson-Shmida, Mourelle, and Williams's beta diversity was also calculated and illustrated in Table 2.

A comparison between rarefaction curves of



(a)



(b)

Fig. 1: Abundance distribution of taxa at different taxonomic levels in (a) Set 1 earthworm gut and (b) Set 2 earthworm gut. The biggest circles depict phylum level, while decreasingly sized circles represent classes, orders, families, genera, and species. Within each level, circle size reflects the taxon's relative abundance. Five most dominant taxa are highlighted in red at the species level, numbered according to their abundance.

both samples showed the reliability and representativeness of the sampling strategy and richness as well as complexity of microbial community. The initial sharp rise of curve followed by a plateau demonstrates that the metagenome has captured most community's diversity with sufficient sequencing depth. It confirms that set 2 possesses greater microbial diversity than set 1.

The targeted loci database from One-Codex platform classified the sequence of set 1 into 30 phyla, 76 classes, 173 orders, 373 families, 794 genera, and 1354 species. For set 2, it was 38 phyla, 100 classes, 220 orders, 478 families, 1101 genera, and 1928 species. The abundance distribution of taxa at different taxonomic levels is shown in Figure 1. Proteobacteria was the highest abundant phyla in both sets. In set 1, Proteobacteria (57.54%) was followed by Actinobacteria (20.87%), Firmicutes (5.6%), and Bacteroidetes (3.13%). The sequence of phyla in set 2 was highly abundant Proteobacteria (59.26%) followed by

Actinobacteria (14.45%), Bacteroidetes (11.21%), and Firmicutes (5.45%).

The Class level identification revealed that Alphaproteobacteria with 33.65% classified reads in set 1 and 36.62% classified reads in set 1. The next highly abundant classes in both sets were Gammaproteobacteria and Actinobacteria. The abundance of both classes was higher in set 1 (20.33% and 20.17%) compared to set 1 (15.71% and 13.23%). Other identified classes having more than 1% reads in set 1 were Bacilli, Clostridia, Flavobacteria, Betaproteobacteria, and Mollicutes with 3.47%, 1.89%, 1.65%, 1.51%, and 1.31% classified reads, respectively. On the contrary, important classes of set 2 were Bacilli (3.39%), Deltaproteobacteria (2.93%), Betaproteobacteria (1.94%), Clostridia (1.82%), Cytophagia (1.2%), and Planctomyceria (0.79%).

Micrococcales, Rhodobacterales, Rhizobiales, and Aeromonadales were the most dominant orders with 18.82%, 15.03%, 13.54%, and 10.01% abundance in set 1. The sequence of highly abundant orders in set 2 was Rhizobiales (16.96%), followed by Micrococcales (11.71%), Rhodobacterales (9.91%), and Flavobacteriales (8.29%). Along with this, Rhodospirillales, Xanthomonadales, Pseudomonadales, and Bacillales were present in both sets with varying abundance.

The highly abundant families were Rhodobacteraceae and Microbacteriaceae, but the abundance level was different. In set 1, Rhodobacteraceae (15.05%) was dominant followed by Microbacteriaceae (13.6%). Set 2 showed Microbacteriaceae as a dominant family continuing with Rhodobacteraceae with 10.04% and 9.73% abundance. The list of common families includes Bacillaceae, Rhizobiaceae, Xanthomonadaceae, Flavobacteriaceae, and Phyllobacteriaceae. Aeromonadaceae and Demequinaceae were unique to set 1 only. Similarly, Hyphomicrobiaceae, Pseudomonadaceae, and Moraxellaceae were limited to set 2.

At the Genus level, *Gemmobacter* was one of the dominant genera with 9.96% and 2.29% classified reads in sets 1 and 2, respectively. *Aeromonas* (9.96%) was highly abundant followed by *Microbacterium* (9.39%) in set 1, while in set 2 *Microbacterium* (5.63%) was the most dominant one. The list of top identified genera of set 1 also includes *Demequina* (3.26%), *Pseudoxanthomonas* (1.34%), *Candidus Bacilloplasma* (1.25%), *Flavobacterium* (1.17%), and *Xanthobacter* (1.11%). The genera lineup of set 2 contains *Pseudomonas* (1.75%), *Actinobacter* (1.54%), *Rhodobacter* (1.35%), *Devosia* (1.35%), *Bacillus* (1.3%), *Pseudoxanthomonas* (1.23%), and *Sediminibacter* (1.09%).

Microbial diversity examination at the species level revealed a deeper understanding of its complexity. The most classified reads were confined to *Gemmobacter aquaticus* (3.66%) and *Microbacterium schleiferi* (1.43%) in sets 1 and 2, respectively. Some other dominant species from set 1 were *Aeromonas hydrophila* (2.1%), *Microbacterium testaceum* (1.93%), *Microbacterium schleiferi* (1.67%), and *Demequina lutea* (1.41%). The dominant species group of set 2 was comprise of *Sediminibacter furfurosus* (1.09%), *Zeaxanthinibacter aestuarii* (1.03%), *Rhodobacter blasticus* (1.01%), and *Marinibacterium profundimaris* (1%). MEGAN screening of these datasets confirms the rich taxonomic diversity observed with One-Codex platform.

A comparative species taxonomic analysis came up with a significant difference in the microbial community at each taxonomic level. Bacteroidetes (11.21%) were more dominant phyla in set 2 compared to set 1, which was only 3.13%. Phyla like Tenericutes and Chlorophyta were only confined to set 1. In case of set 2, it was Balneolaeota and Chlamydiae. When it comes to class level, abundance of class Flavobacteriia was 4.5 times higher in set 2. Class Mollicutes was foremost in set 1. Conversely, Gemmatimonadetes were predominant class in set 2. Aeromonadales have 9.8% classified reads in set 1, but it decreases to only 0.17% in set 2. Flavobacteriales and Sphingomonadales compensate for the decrease

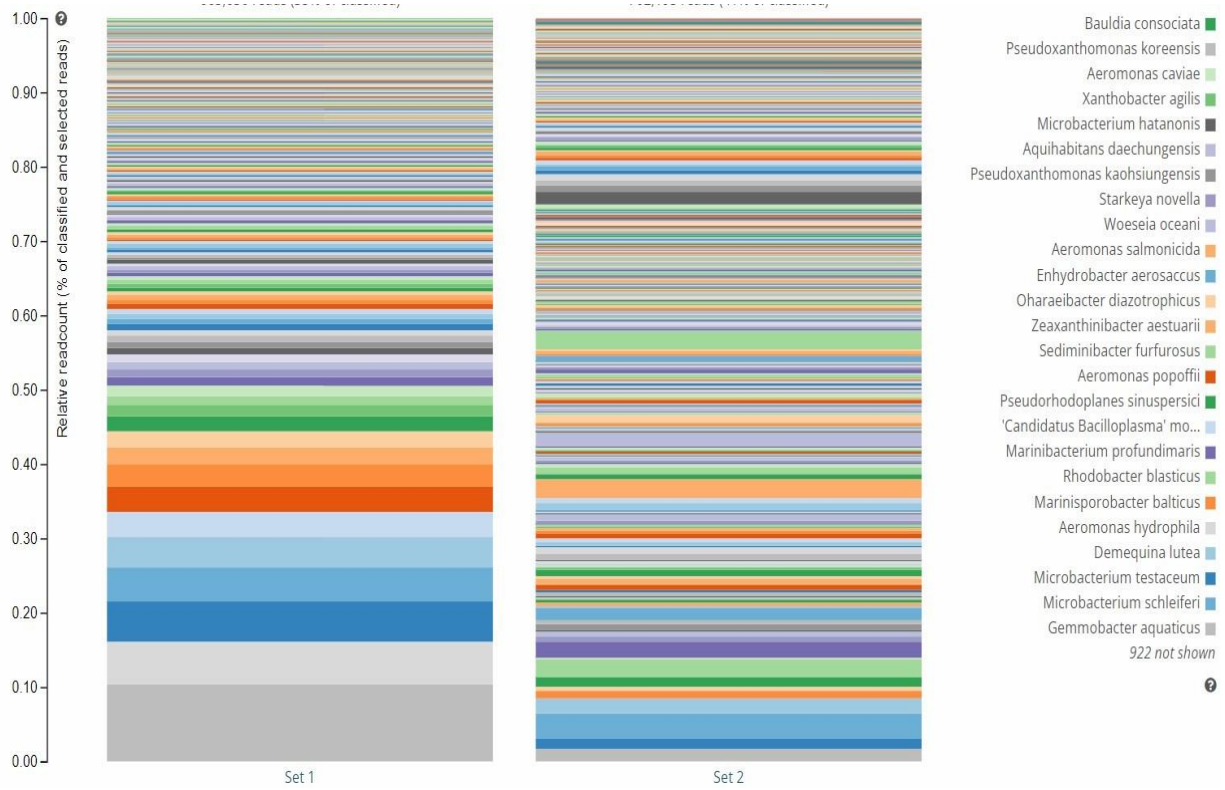


Fig. 2: Comparative taxonomic analysis at Species level.

in set 2. Family-level comparison showed a significant difference in abundance of Aeromonadaceae. It was 9.8% for set 1 and just 0.17% for set 2. As class Flavobacteriia was higher in set 2, one of its families Flavobacteriaceae was prevalent in set 2. Genera *Aeromonas*, *Gemmobacter*, *Microbacterium*, and *Demequina* take over major classified reads of set 1. *Acinetobacter*, *Sediminibacter*, *Chryseobacterium*, and *Zeaxanthinibacter* cover the spaces of these genera in set 2. A detailed comparative analysis at species level is presented in Figure 2. A species-level comparison gave insights of genus level comparison between two sets. *Aeromonas hydrophila, Aeromonas popoffii, Aeromonas salmonicida, Marinisporobacter balticus*, and *Oharaeibacter diazotrophicus* were restricted to set 1. On the other hand, the limited species list of set 2 includes *Sediminibacter furfurosus, Rhodobacter blasticus, Zeaxanthinibacter aestuarii, Marinibacterium profundimaris*, and *Woeseia oceani*.

The taxonomic verification of both metagenomes was additionally corroborated with STAMP analysis. It confirms that Proteobacteria and Actinobacteria were significantly over-represented phyla in both sets (Fig. 3). The comparative STAMP analysis was executed with a statistical confidence threshold of 95% revealing that Bacteroidetes, Streptophyta, Acidobacteria, Balneolaeota, Chlamydiae, and Planctomycetes were highly abundant phyla in set 2. It was replaced by Actinobacteria, Tenericutes, Firmicutes, Chlorophyta, and Rhodophyta in set 1.

Taxonomic to phenotypic mapping of data was performed using web-based server METAGENassist. The classification of microbes was achieved on the basis of metabolism, biotic relationships, and energy sources. The metabolic composition of both sets is given in Figure 4. The major identified significant metabolic pathways were Sulfate reducer, Ammonia oxidizer, and Nitrite reducer in both sets. Some other leading

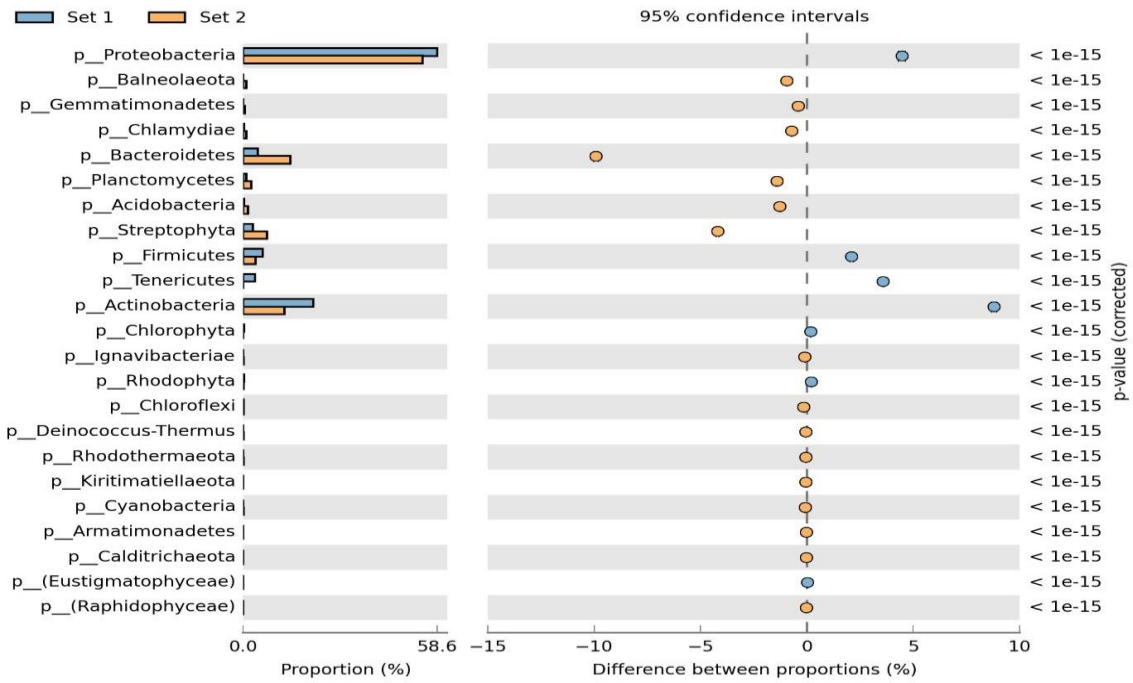


Fig. 3: Comparative phylum level analysis using STAMP at 95% confidence interval between set 1 and set 2.

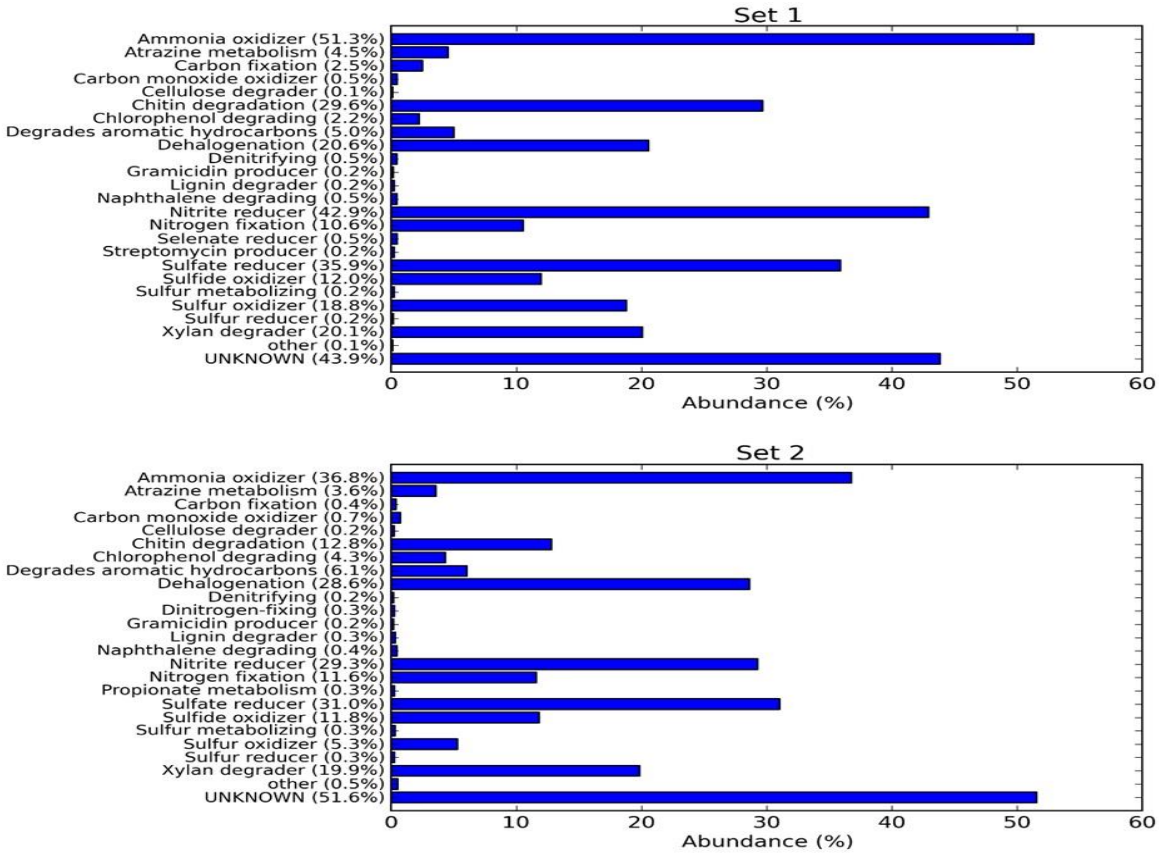


Fig. 4: Functional analysis (Metabolism) with % abundance using METAGENassist.

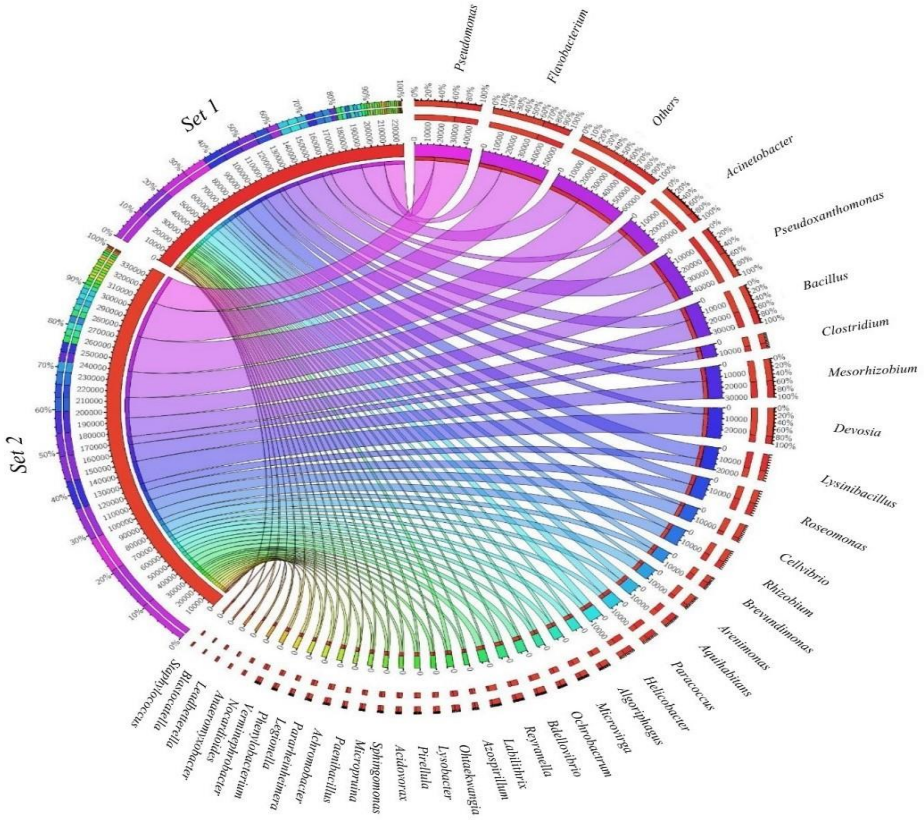


Fig. 5: Circos plot showing core genera among two sets.

metabolic groups in set 1 were Chitin degradation (29.6%), Dehalogenation (20.6%), Xylan degrader (20.1%), and Nitrogen fixation (10.6%). In set 2, the abundance of these metabolic groups was changed to 12.8%, 28.6%, 19.9%, and 11.6%, respectively. Selenate reducer and streptomycin producer were only confined to set 1, while Propionate metabolism was limited to set 2. Common metabolic pathways with lower abundance include Naphthalene degrading, Lignin degrader, degrades atomic hydrocarbons, Chlorophenol degrading, Atrazine metabolism, and Gramicidin producer.

In terms of energy sources, the abundance of Heterotrophs, Autotrophs, Organotrophs, Organoheterotrophs, Diazotrophs, Phototrophs, Chemoorganotrophs, and Methylotrophs was significant. Heterotrophs were highly dominant with 8.5% and 17.8% in sets 1 and 2, respectively. The abundance of Chemoheterotrophs, Chemolithotrophs, Diazotrophs, Phototrophs, and Methanotrophs in set 1 was 0.4%, 0.02%, 0.7%, 0.6%, and 0.02%, respectively. It was increased to 1.3%, 0.2%, 1.5%, 1.4%, and 0.4% in set 2, respectively. When it comes to biotic relationships, free-living bacteria rule over symbiotic ones. The abundance of free-living bacteria was 83.7% and 64.1% in sets 1 and 2, respectively. In case of symbiotic bacteria, abundance was approximately doubled in set 2 (17.4%) than in set 1 (9.3%).

Core Microbiome

Core microbiome analysis was performed using MEGAN. As per the results, 56.52% of OTUs were shared among both sets. Sets 1 and 2 possess 19.44% and 24.03% unique OTUs. The key phyla in core microbiome were Proteobacteria, Bacteroidetes, Actinobacteria, and Firmicutes. Alphaproteobacteria, Gammaproteobacteria, and

Betaproteobacteria were the most prevalent classes of Proteobacteria. The list continues with Actinomycetia, Flavobacteria, Vicinamibacteria, Bacilli, and Clostridia. The major identified core families were Flavobacteraceae, Rhodobacteraceae, Xanthomonadaceae, Microbacteriaceae, and Vicinamibacteraceae.

Core genera are represented in form of circos plot (Figure 5). The predominant genus was Flavobacterium with 9.63%. It was followed by *Pseudomonas*, *Pseudoxanthomonas*, *Acinetobacter*, *Bacillus*, *Mesorhizobium*, and *Devosia* with 7.94%, 7.48%, 5.86%, 5.53%, 5.36%, 4.98%, respectively. *Microbacterium schleiferi, Marinisporobacter balticus, Gemmobacter aquaticus, Demequina lutea, Marinibacterium profundimaris*, and *Aquihabitans daechungensis* were the top core species of earthworm gut microbiome.

Discussion

Bacterial community study during vermicomposting of coconut leaf, Grape Marc, Dewatered Sludge, Winery Waste, Silver Wattle, etc. was carried out by researchers to identify microorganisms responsible for degradation of substrate (Gopal *et al*., 2017; Gómez Brandón *et al*., 2019; Zhang *et al*., 2020; Rosado *et al*., 2021; Karapantzou *et al*., 2023). Effect of pesticides, feed materials, Zn nanoparticles, arsenic, vanadium stress, and Hg Contamination on earthworm gut microbial community has also been carried out in past few years. The current study gives insights into microbial degradation of a complete waste product which is secondary sewage sludge (SS). As SS is the final product of a wastewater treatment plant, all water pollutants as well as volatile solids, heavy metals, and pathogens are also transferred to SS (Azarmanesh *et al*., 2020). When SS is fed with WH to earthworms, it turns out into a valuable vermicompost. The results revealed that microbial community present in earthworm gut is responsible for this degradation with formation of value-added vermicompost.

The diversity matrix and rarefaction curve analysis cleared that species diversity is higher in set 2. Evenness index is also higher in set 2 indicating distribution of species is more even compared to set 1. The lower value of Dominance index and Berger-Parker for both sets show less dominance of particular species. Menhinick's and Margalef's richness index specifies that set 1 has higher species richness. According to Mulya *et al.* (2021), these indices give weightage of number of species. Besides this, Shannon diversity index represent weightage of both dominant and rare species, while Simpson diversity index represent weightage of only dominant species (Hubalek, 2000). This explains that the variation in indices is because of different number of species in the sets. Different measures of beta diversity show moderate dissimilarities between two sets. From the diversity matrix, it can be inferred that same feed material can also alter gut microbial diversity.

Taxonomic metagenomic analysis showed that Proteobacteria is the most prevalent phylum in both sets. Microbes belonged to Proteobacteria are generally involved in metabolic processes like aromatic hydrocarbons, naphthalene, dye, pesticide, and oil degradation, sulfur reduction, as well as nitrogen fixation. The process explains a significant role of Proteobacteria in organic matter and waste degradation. Previous studies of earthworm gut microbiome also identified Proteobacteria as the dominant phylum (Liu *et al*., 2018; Thakur *et al*., 2021; Astaykina *et al*., 2022). Other important phyla were Actinobacteria, Firmicutes, and Bacteroidetes. Actinobacteria are experts in degradation of cellulose and lignin (Lewin *et al*., 2016). Bacteroidetes were normally able to degrade xylan, pectin, biopolymers, and other complex organic compounds with symbiotic ability (Thomas *et al*., 2011). Majorly SS contains polycyclic aromatic hydrocarbons (PAH), dioxins, polychlorinated biphenyls (PCB), pharmaceutical residues, furans, detergent, personal care products, synthetic steroids, heavy metals, and endocrine disruptors (Hudcová *et al.*, 2019; Pola *et al*., 2021). On the other hand, WH can be considered as lignocellulolytic biomass. That explains the higher abundance of Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes. An

experiment was done to observe the microbial community during Dewatered Sludge vermicomposting by Zhang *et al.* (2020). During the experiment, Proteobacteria, Actinobacteria, and Bacteroidetes were predominant. Similar results were also noted by Gómez Brandón *et al*. (2019) and Domínguez *et al.* (2019) during the vermicomposting of Grape Marc and Scotch broom.

Gemmobacter sp., a predominant species of set 1 can degrade organic components like toluene, pyridine, and methylene chloride (Liu *et al*., 2020). The other dominant species were *Microbacterium schleiferi* and *Microbacterium testaceum*. *Microbacterium schleiferi* can degrade 1,3,5-trimethylbenzene, one of the organic compounds present in sewage sludge (Lv and Yu, 2014; Santana *et al*., 2021). *Microbacterium testaceum* can produce IAA and auxin, plant growth promoting hormones. So, the composted feed can be used as a potting amendment for plants. The highly abundant species of set 2 was *Sediminibacter sp.* The active substrate for this organism is galactomannan, which is present in WH (Issa, 1988; Beidler *et al*., 2023). The next predominant species were *Zeaxanthinibacter sp*. and *Rhodobacter blasticus*. The main functions of *Zeaxanthinibacter* are zeaxanthin pigment production, sulphur metabolism, and secondary metabolite production in plants with antifungal activity (Efremova, 2023). *Rhodobacter blasticus* is involved in removal of heavy metals like Zn and Cu (Sayadi and Nourzadeh, 2018) as well as it can decrease the concentration of organic compounds (Wen *et al*., 2016).

The feed composition of set 1 was 75% SS + 25% WH. The higher abundance of *Gemmob sp*., *Microbacterium schleiferi,* and *Microbacterium testaceum* was because of higher amount of SS. These species are majorly involved in aromatic organic compounds mainly present in SS. When it comes to set 2, the feed composition was 50% SS + 50% WH. The dominant species (*Sediminibacter sp., Zeaxanthinibacter sp*., and *Rhodobacter blasticus*) are engaged in degradation of galactomannan, organic compounds, plant metabolisms, and removal of heavy metals. So, a higher amount of WH increased the abundance of these species.

The taxonomic to phenotypic profiling of earthworm gut exhibits that earthworm gut can be viewed as a microenvironment, where a complete sulfur and nitrogen metabolism is taking place. Aside from these, degradation of lignocellulolytic biomass like cellulose, chitin, lignin, and xylan as well as bioremediation processes like Chlorophenol, naphthalene, and atomic hydrocarbon degradation along with Atrazine metabolism and Dehalogenation also occurs within the gut. Microbes associated with dehalogenation process were predominant. This process increases bioremediation of organic wastes by removing halogens from organic compounds. The composition of SS includes 0.3-2.3% sulfur and sulfates (Dewil *et al*., 2008). Hence, sulfur oxidizers, and reducers are more in set 1 as it contains high amount of SS than set 2. On the contrary, cellulose and lignin degraders are higher in set 2 because of high WH content. The analysis confirms that microbes having boundless functions such as biodegradation, bioremediation, biogeochemical cycles, etc. are present in the earthworm gut. The presence of such organisms elucidates brisk degradation of pollutants, lignocellulolytic biomass, and organic matter. Nitrogen-fixing microbes are responsible for a nitrogen-rich end product, beneficial for plants.

Core microbiome analysis discloses that 56.52% of OTUs are shared between two sets. Although both sets having some unique organisms, core microbiome remains intact. A study by Liu *et al*. (2018) showed that 21.22% of OTUs are common in earthworms fed with three different diets. A similar study by Aira *et al.* (2019) came up with 5% shared bacterial taxa among aging casts (0, 7, 15, 30, and 60 days age) of earthworms *Aporrectodea caliginosa*. These organisms construct the core microbiome of earthworm cast. In present study, *Pseudomonas*, *Flavobacterium*, *Acinetobacter*, and

Psudoxanthomonas are the highly dominant genera. Along with these, some nitrogen-fixing genera like *Rhizobium, Mesorhizobium, Labilithrix,* and *Azospirillum* are also present. *Labilithrix,* and *Azospirillum* are not only related to nitrogen fixation but also related to organic matter degradation and auxin production (Steenhoudt and Vanderleyden, 2000; Zou *et al*., 2022). Other plant hormone producing genera is *Lysinibacillus*, which is also responsible for organic pollutants and heavy metal remediation (Pantoja-Guerra *et al*., 2023). *Sphingomonas* is associated with IAA and gibberellins production can also degrade organometallic compounds (Asaf *et al*., 2020). Polysacahharides and biomass-degrading organisms were *Algoriphagus* and *Microvirga*, respectively (Alegado *et al*., 2011; Jiménez-Gómez *et al*., 2019). Genera *Nocardioides* and *Anaeromyxobacter* have a good ability to remediate hydrocarbons, halogenated compounds, and herbicides (Freitas *et al*., 2008; Mitzscherling *et al*., 2022). One atrazine-degrading organism is *Arenimonas* present with lower abundance. A distinct genus of lumbricid earthworm (*Eisenia fetida*) is *Verminephrobacter*, a symbiont with nephridia of earthworms. This is a key genus of earthworm core microbiome.

The core microbiome study revealed that earthworm gut owns a microbial community with a number of functions important for hydrocarbon degradation, lignocellulolytic biomass degradation, nitrogen fixation, biogeochemical cycles, production of plant growth hormones, phosphorus stabilization, organic matter decomposition, aromatic herbicides, and other pollutant destruction. This unique microbial community makes earthworm gut capable of different waste decomposition and production of vermicompost, rich in nutrients and plant growth promoters.

Conclusion

The community metagenomic screening of earthworm gut exposed the bacterial community responsible for decomposition of feed material and the conundrum behind the transformation of Secondary sewage sludge- a complete waste product into vermicompost. The results also indicate that the bacterial community changes according to feed material. The same feed in different proportions alters the gut microbiome. Even though, a core bacterial community remains the same in different conditions. The core microbiome includes bacteria having numerous functions crucial for biogeochemical cycles, herbicides, aromatic hydrocarbon, and other pollutant destruction, lignocellulolytic biomass and organic matter degradation, phosphorus stabilization, nitrogen fixation, and plant growth hormones production. This elucidates that earthworm gut microbiome changes with feed material and converts it into vermicompost.

Ethical Statement

This study was in accordance with the Research Advisory Committee (RAC) of the Veer Narmad South Gujarat University, Surat, Gujarat. The present study was a continuation of the research project funded by the Gujarat State Biotechnology Mission (GSBTM).

Conflict of Interest

The authors declare no conflicts of interest.

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