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ORIGINAL PAPER

Changes in structure and function of bacterial communities during coconut leaf vermicomposting

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Abstract To understand bacterial community dynamics during the vermicomposting of lignin-rich coconut leaves using an indigenous isolate of an epigeic earthworm, *Eudrilus* sp., we employed amplicon-based pyrosequencing of the V1 to V3 region of the 16S rRNA genes. Total community DNA was isolated from two separate vermicomposting tanks in triplicate at four different stages of the process: pre-decomposition (15th day), initial vermicomposting (45th day), 50–70% vermicomposting (75th day) and mature vermicompost (105th day). Alpha diversity measurements revealed an increase in bacterial diversity till the 75th day, which then declined in the mature vermicompost. Beta diversity comparisons showed formation of distinct, stage-specific communities. In terms of relative abundance, the Acidobacteria, Actinobacteria, Chloroflexi, Gemmatimonadetes, Nitrospirae, Planctomycetes, TM7 and WS3 groups increased until the 50–70% vermicomposting stage ($p = 0.05$). During the same time, the abundance of Bacteroidetes and Proteobacteria decreased. In contrast, the levels of Firmicutes increased throughout the 105-day vermicomposting process. The distribution of the most abundant OTUs revealed that each stage of the vermicomposting process possessed its own unique microbiome. Predictions based on the OTUs present by PICRUSt suggested a functional shift in the microbiome during vermicomposting. Enzymes and pathways of lipid and lignin metabolism were predicted to be initially abundant, but by the end of the process, biosynthesis of secondary metabolites and plant beneficial properties were enriched. The study

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revealed that bacterial communities undergo a continuous change throughout the vermicomposting process and that certain OTUs associated with specific stages could be targets for further improvements in the process.

Keywords 16S rRNA · Coconut leaf · Lignin degradation · Metagenomics · Pyrosequencing · Vermicompost

Introduction

Vermicomposting is a viable and cost-effective waste recycling technology in which organic waste matter is converted to useful compost through the combined actions of microorganisms and earthworms (Dominguez 2011). The resultant vermicompost is then used as manure for improving the physical, nutritional and biological properties of soil (Chaoui et al. 2003; Arancon et al. 2006; Lazcano and Dominguez 2011). The earthworm species most often used for vermicomposting include tiger or red worms (*Eisenia foetida*), red wrigglers (*Lumbricus rubellus*), African night crawlers (*Eudrilus eugeniae*) and the Indian blue worm (*Perionyx excavatus*). These epigeic species are rarely found in soil as they are adapted to the special conditions in rotting vegetation and compost and manure piles (Kale 1998). Using these species, a wide range of agricultural and horticultural wastes have been converted to vermicompost (Edwards and Fletcher 1988; Jeyabal and Kuppuswamy 2001; Garg et al. 2006; Suthar 2008; Vivas et al. 2009; Raphael and Velmourougane 2011). However, these species are typically ineffective in degrading lignin-rich organic waste, which is a major by-product of coconut production in the southern states of India.

The coconut palm (*Cocos nucifera* L) is an important oilseed crop cultivated in more than 80 tropical countries. India is one of the leaders in coconut production, with approximately 2 million hectares under cultivation, providing the livelihood for 10 million Indians (http://coconutboard.nic.in/stat.htm). Coconut farming annually generates 6–8 tonnes of leaf biomass waste per hectare. The high lignin content of 30–40% makes the coconut leaves and petioles very recalcitrant to decomposition, and 18 months are required for their unassisted degradation (Thomas et al. 1998; Upadhyay et al. 1998). However, vermicomposting with a local isolate of earthworm *Eudrilus* sp. hastens the degradation of the coconut leaves to 90 days (Prabhu et al. 1998). The coconut leaf vermicompost also contains high levels of plant-beneficial microorganisms, and its application not only improves the soil microbial biomass and fertility but also significantly increases the coconut yield (Gopal et al. 2010; Maheswarappa et al. 2014).

Microorganisms associated with the substrate and earthworm play key roles in vermicomposting (Dominguez 2011). They not only help in the bio-oxidation during vermicomposting but also during the ageing of the vermicompost once the earthworms have been removed (Aira et al. 2007). Microorganisms in different types of vermicomposts have been studied widely for (i) their contribution to the quality of vermicomposts (Fracchia et al. 2006; Aira and Dominguez 2011; Grantina-Ievina et al. 2013), (ii) their abundance and diversity during different stages of the vermicomposting process (Vivas et al. 2009; Gomez-Brandon et al. 2011, 2012; Chen et al. 2015), and (iii) the levels of plant-beneficial microorganisms in the finished vermicompost (Gopal et al. 2009; Raphael and Velmourougane 2011; Pathma and Sakthivel 2013). These studies generated limited information on the diversity of microbes involved in vermicomposting of different substrates. However, no information is available on their role in coconut leaf vermicomposting. Such information would benefit improvement programs to enhance the efficiency and yield of the process.

To gather this information, amplicon-based pyrosequencing of the V1–V3 regions of the 16S rRNA genes was employed to elucidate the bacterial community dynamics during different stages of coconut-leaf vermicomposting by an epigeic earthworm, *Eudrilus* sp. We hypothesized that the diversity of microbes would increase initially due to the availability of wider range of nutrients and as the vermicomposting process nears completion, members with specialized metabolic functions would selectively dominate the community, thereby leading to a decrease in the overall diversity in mature vermicompost. Our observations generally supported this hypothesis and also revealed specific associations between microbes at different stages of the vermicomposting process. Imputed metagenomics further identified specific changes in the functional capacity of the microorganisms that were likely to be involved during different stages of vermicomposting.

Materials and methods

Coconut leaf vermicompost production

The process of vermicomposting of coconut leaves using an indigenous isolate of African night crawler, *Eudrilus* sp., was developed at the Central Plantation Crops Research Institute, Kerala, India (Prabhu et al. 1998). Vermicomposting was carried out as a batch system in cement tanks located at a farm within the institute premises (12°30′N and 75°E, 10 m above MSL; approximately 250 m away from the Arabian Sea (Western Coast of India)). The tanks were covered with a slanting roof made of thatched coconut leaves to prevent sunlight and rainwater from falling directly into them. Two cement tanks, 9 m long × 2 m wide × 1 m deep, subdivided into three 3-m long compartments by brick and mortar walls were used for this study. Each 3 m compartment within the two tanks accommodated 300 kg of the coconut leaves and was considered one of six replicates.

The entire vermicomposting process took 105 days, including the 15-day pre-decomposing stage (Fig. 1). To begin, the bed of each tank is prepared using senescent, dried and fallen coconut leaves of the West Coast Tall variety. Each frond weighed 4–5 kg and stood 2–2.5 m tall. The fronds were cut into two and stacked to about one-third of a meter height inside each tank. A thin layer of cow dung slurry, obtained from the Institute dairy unit, was spread over this. On a wet weight basis, the ratio of coconut leaves to cow dung slurry was 10:1. Two more layers of chopped coconut leaves and cow dung slurry were added to each tank, above which a layer of dried grass or coconut leaves was spread as mulch. The tank was then covered with a greenhouse shade net to prevent entry of rodents and rhinoceros beetles. During this pre-composting stage, water was added at regular intervals for 15 days to maintain the moisture content at 40–50%. Coconut-leaf degrading earthworms, *Eudrilus* sp., were added at day 16 at a ratio of one adult worm per kg of coconut leaves to initiate the second stage. Water was regularly added to maintain the moisture content at 40–50%. As the earthworms consumed the substrate and converted it to vermicompost, the level fell over 30 days from the time of earthworm addition to about 75% of the initial height, marking the end of the second stage. During the third stage, which lasted until the 60th day since earthworm addition, approximately 50–70% of the substrate was already converted to vermicompost and the level fell to about 50% of the initial height. At that time, the water application was stopped and the compost was heaped at the centre of the tank, marking the beginning of the maturation stage. During this stage (typically starting on the 76th day), the vermicompost was sorted to remove the earthworms and un-decomposed material, and allowed to air-dry. Finally, it was removed from the tank on the 90th day since earthworm addition and passed through a 2 mm sieve to form the mature vermicompost.

Sample collection

Samples were collected from the two tanks (VA and VB) in triplicate (one from each compartment) during all four stages of the coconut leaf vermicomposting process. Specifically, (i) on the 15th day upon completion of pre-decomposition stage just before the addition of earthworms (VA_{15} and VB_{15}); (ii) on 45th day, 30 days after addition of earthworms during the second stage (VA_{45} and VB_{45}); (iii) on 75th day, 60 days after addition of the earthworms when conversion of more than 50% substrates to vermicompost was complete (VA_{75} and VB_{75}); and (iv) on 105th day, 90 days after inoculation of the worms when mature, sieved coconut leaf vermicompost was ready (VA_{105} and VB_{105}) (Fig. 1). At each sampling time, approximately 0.5 kg of material were collected from ten randomly chosen places and depths of each sub-tank to obtain 5 kg. This material was pooled and homogenized for each compartment, and the DNA was immediately extracted. In addition, 20 adult earthworms were collected from randomly chosen places and depths in each compartment during the 75th (EA_{75} and EB_{75}) and 105th days (EA_{105} and EB_{105}). Collected earthworms were washed three to four times with sterile distilled water and then transferred to 70% ethanol until they turned rigid. They were then dissected and their gut contents were extracted. The gut contents of all worms from a single compartment were pooled, and the resulting ∼50 g of biomass was processed immediately for DNA extraction. Thirty-six samples were thus obtained: 24 of vermicompost

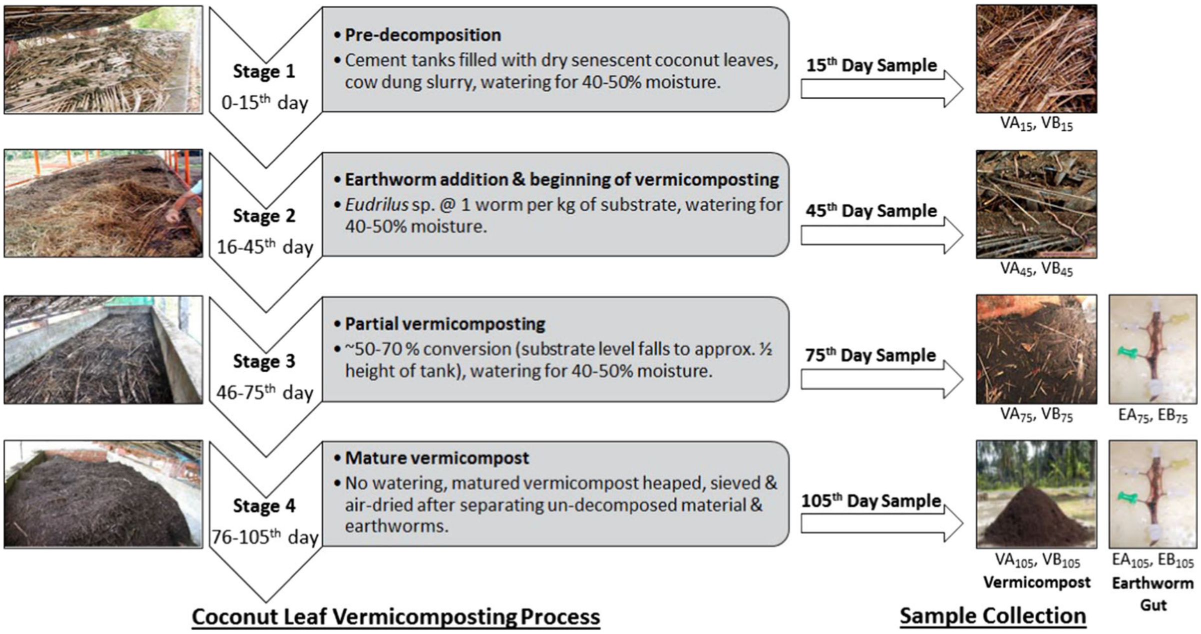

Fig. 1 Coconut-leaf vermicomposting process and sampling time points. Images shown are actual site photographs taken during sampling at the specified time points during the four stages of the vermicomposting process

(three compartments from each of the two cement tanks at four time points) and 12 of earthworm gut contents (three compartments from each of the two cement tanks at two time points).

Chemical and nutrient analysis of substrate and mature vermicompost

Pre-decomposed substrate and vermicompost samples were analysed for various chemical and nutrient properties. Because there was little variation in the chemical composition of substrate used (Garg et al. 2006) and the composting process between tanks involved the same starting substrate material (Gopal et al. 2010), only the samples from one tank were analyzed. pH was analyzed in a 1:2.5:solid:water ratio (w/v) slurry using a pH meter (Eutech Cyberscan, USA). Oven-dried samples were ground for estimation of phosphorus and potassium (Jackson 1967). Total nitrogen in the samples was estimated using a N-analyser (Gerhardt Vapodest, UK) by the Kjeldahl method (1883). Total phenols were estimated by the Bray and Thorpe method (1954) using Folin–Ciocalteu reagent. Humic acid was estimated at the Centre for Applied Research and Development, Neyveli Lignite Corporation, India by a proprietary ISO 2000 method described by International Humic Substances Society.

Genomic DNA extraction, amplicon preparation and pyrosequencing

All samples were immediately subjected to genomic DNA extraction with the MoBio Power Soil DNA kit (MoBio, Carlsbad, USA) based on the manufacturer's instructions. The quality and concentrations of the genomic DNA was ascertained using a Nanodrop spectrophotometer and 0.8% agarose gel electrophoresis. The DNA was then stored at −20 °C for further studies. In addition, a set of three previously cloned full length 16S rRNA genes served as controls to test for errors during PCR amplification and pyrosequencing as described by Garcia et al. (2011).

PCR amplification of the V1–V3 region of the bacterial 16S rRNA genes, amplicon cleanup and pooling of purified amplicons for pyrosequencing was carried out as described earlier (Garcia et al. 2011). Specifically, 25-cycle amplification was conducted using primers 515R-NK and 27F-YM+3 as shown: Adaptor(A)-BARCODE-(515R-NK) and Adaptor(B)-(27F-YM+3) for the Roche 454 GS FLX titanium platform as described in Garcia et al. (2011). Gel quantification of amplicons was done on a Typhoon Trio + Variable mode imager (GE Healthcare, Pittsburg, Pennsylvania) using Image Quant 5.2 (Molecular Dynamics). Amplicons from replicates were pooled in equimolar concentrations prior to gel purification using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Orange, California). Concentration of the purified DNA was confirmed using the Experion System (Bio-Rad, Hercules, California), and pooled amplicons were further purified using the Agencourt AMpure magnetic beads (Beckman Coulter, Brea, California). The purified DNA was submitted to the Georgia Genomics Facility (http://dna.uga.edu/) for pyrosequencing. The sequences were submitted to SRA with accession number SRP107165.

Sequence analysis

Sequences were processed using an analysis pipeline that combined both QIIME v1.9.1 (Caporaso et al. 2010) and MOTHUR v1.32.1 (Schloss et al. 2009). We chose to use a 400 bp sequence length cutoff for quality trimming because it provided more taxonomic information compared to a 250 bp cutoff used previously (Garcia et al. 2011). Demultiplexed and quality-filtered sequences were subjected to operational taxonomic unit (OTU) picking using close reference protocol against the Greengenes database at 97% sequence similarity cutoff (DeSantis et al. 2006) followed by selecting the representative sequence from each of the OTUs for downstream analysis. The taxonomy of each of these representative sequences was assigned using the RDP classifier (Wang et al. 2007) and the QIIME-compatible biom table was constructed. It has been proposed recently that the practice of data normalization and rarefaction to the smallest sample size is statistically inefficient and, therefore, inadvisable (McMurdie and Holmes 2014). In line with this proposal, the data were not normalized for comparisons. However, the calculation of alpha diversity indices was carried out using both the complete dataset as well as a subsample of equal size using the Ace, Chao1, observed species, and Shannon and Simpson diversity indices. Weighted and unweighted UniFrac beta diversity indices were also calculated (Lozupone et al. 2011). For the latter and for calculating significant differences between samples, singleton OTUs were removed from the analysis. In addition, *K*-shuff was used to calculate the intra- and cross-*K*-function as measures of structural and compositional diversity, respectively (Jangid et al. 2016). This method is based upon distance matrices and considers both the sequence diversity and abundance in distinguishing community structures.

The amplicon sequences were analyzed using PICRUSt (Langille et al. 2013) to predict the functional content of the bacterial communities during vermicomposting. For the earthworm gut, only one sample (EB_{75}, replicate 2) yielded sufficient reads and hence, dataset from this was randomly sampled thrice to obtain enough sequences for comparison to the vermicompost sequences. Results obtained from PICRUSt were plotted in the form of a cladogram using the online tool for linear discriminant analysis effect size (LEfSe) available at https://huttenhower.sph.harvard.edu/galaxy/ (Segata et al. 2011). LEfSe allows the identification of pathways characterizing the differences between different stages of the vermicomposting process as well as the earthworm gut bacterial community.

Results and discussions

Analysis of the 16S rRNA gene controls

To evaluate the reliability of the analysis pipeline, controls were constructed comprising of PCR amplicons of either single 16S rRNA genes or mixtures of 16S rRNA genes. The mol% G+C content of the genes ranged from 33 to 70% to determine the bias against high G+C genes (Table S1). We obtained 1722 good quality sequences that ranged from 451 to 517 bp in length at an average of 287 sequences per control (Table S1). While the controls used only three 16S rRNA genes, they generated 92 OTUs at $D = 0.03$ (365 OTUs at $D = 0.01$), slightly higher than those obtained by Garcia et al. (2011) for the same controls from a different sequencing reaction. However, the number of correctly classified sequences was much higher (97.38–100%) compared to the 93.83% observed previously, suggesting that the rate of incorporation of artificial errors during PCR amplification or sequencing was much lower for this study. Of particular concern for environmental studies, the number of incorrectly assigned sequences was higher in the mixed template than the single template controls (Table S1). Most of the spurious OTUs grouped into only two OTUs and were taxonomically assigned to the 'Unclassified' group. Because sequencing errors would have likely generated singletons, these results suggested that the spurious sequences were formed during the PCR amplifications. In contrast, GC content had little effect on the number of sequences obtained and their classification. For instance, both the number of sequences obtained as well as the number of correctly classified sequences was only marginally higher for the control that had higher molar ratios of the high G+C actinobacterial DNA among the mixed templates.

These sequencing controls provided an opportunity to calculate the error rates and standardize the analyses pipeline. However, this approach has been severely overlooked. For instance, based on keyword search at NCBI Pubmed, of the ~1800 publications using pyrosequencing, only ~140 included "pyrosequencing controls". Further examination revealed that only a few of these referred to sequencing template controls similar to those described here. Based on the analysis of controls, it was evident that the number of OTUs can be greatly overestimated and most of the incorrectly classified sequences were grouped into rare OTUs reflecting PCR or sequencing errors. Although the per base error rate for pyrosequencing is comparable to Sanger sequencing (Huse et al. 2007), ~15–30% sequence reads have one or more errors (Kunin et al. 2010); this intrinsic error rate is very high and especially problematic for estimating the rare biosphere.

Description of the bacterial 16S rRNA gene data

Although DNA was successfully extracted from all vermicompost samples, 11 of the 12 earthworm samples yielded very little community DNA and could be PCR amplified only upon cleaning and concentrating the DNA using the MoBio DNA Clean-up kit. Further, only 22 of the 36 samples generated sufficient sequencing reads. Sample VB_{15} (replicate 1), two replicates of VB_{105} (1 and 2), and 11 of the 12 earthworm gut samples (except EB_{75}, replicate 2) did not yield any sequences. Pyrosequencing of the bacterial 16S rRNA genes from these 22 samples yielded 257,031 reads with per sample reads ranging from 1339 to 33,961 (median = 10,009), which upon quality trimming yielded a total 156,039 sequences. These good quality sequences were processed further for phylotype picking and the removal of singletons, yielding 44,855 sequences that clustered into 2522 OTUs. Each sample was represented by 261 to 6636 (median = 1752) sequences forming 123 to 745 OTUs per replicate. The most abundant OTU was represented by 1555 sequences, accounting for 3.5% of the entire sequence data set. The top 20 and 250 OTUs represented 29.4 and 73.5% of the entire sequence data set, respectively.

Occasionally, replicate environmental samples are known to behave unexpectedly different from the other replicates, and it is not unusual for a few samples to yield insufficient number of sequences even after a very conservative PCR strategy and accurate equimolar pooling. For instance, of the 360 samples processed for the 'Wild Life of Our Homes' project, 38 samples did not yield a sufficient number of reads and were not considered for downstream analysis (Dunn et al. 2013). Of the nine locations studied from within 40 homes, the study eventually examined samples from only 19 homes based on a normalized dataset. In concordance with McMurdie and Holmes (2014), we employed a normalization-free approach and used the entire dataset because the current statistical toolbox has the capacity to factor in the effect of unequal size. Further, the sample preparation strategy we employed ensured that replicate samples in our study generated similar number of sequences and were not statistically different.

Reproducibility of the vermicomposting process across the tanks

Before making observations on the community change, the reproducibility of the vermicomposting process across the two tanks was examined. As has been shown earlier (Gopal et al. 2010), with the same substrate, the biological variation between tanks was small. Similar minor variations were also observed between experimental pots undergoing vermicomposting of maize stover by *E. fetida* (Chen et al. 2015). First, the coconut leaf fronds were collected from a monitored coconut plantation implying that the composition of the fronds was very similar. Secondly, the variation of chemical and nutrient parameters within the triplicates was small (Table 1) and in agreement with those reported earlier (Gopal et al. 2010). There was a consistent increase in pH during vermicomposting. Likewise and as is characteristic of coconut leaf vermicomposting, while the humic acid content increased, the amount of potassium and total phenols decreased with increasing decomposition of the substrate. Altogether, these results suggested that the process was reproducible among the replicates.

Similarly, the bacterial communities changed consistently across the two tanks during vermicomposting. There was only a small amount of variation between the tanks for the alpha diversity measures when the dataset was normalized for sample size (Table 2). Similarly, the variation between tanks was small when the entire dataset was analyzed (Table S2). Moreover, the triplicates from within each tank were consistently similar (data not shown). Based on the analysis of the intra-k-functions (I_{kf}), which is a measure of the structural diversity within a sample, the I_{kf} values for samples from the two tanks collected at the same time were not statistically different, and the I_{kf} values differed significantly only between samples collected at different time points (Table 2). Even though some replicates did not yield many reads, differences in sample size were unlikely to have an effect on the I_{kf} values. Indeed, the intra-K-function is a robust metric and not greatly affected by differences in sample size (Jangid et al. 2016). Thus, on the basis of measurements of both chemical and nutrient properties and bacterial community diversity, the vermicomposts and the rRNA gene libraries generated from them were reproducible. Thus, for many subsequent analyses, sequences from the two tanks were pooled to maximize sample coverage.

Community change during coconut leaf vermicomposting

As hypothesized, the bacterial diversity increased till the 75th day, but then declined significantly by the 105th day. While this trend was statistically significant in the Simpson's index, it was only marginally significant in other indices for the complete dataset (Table S2; Fig. 2a). Likewise, the indices showed similar trends when calculated using a normalized dataset of 250 sequences per replicate suggesting that the variation was not due to differences in sample size, to which some alpha-diversity indices are known to be sensitive (Table 2). A similar trend was also observed in the rarefaction curves for the samples (Fig. S1), which showed increased diversity on the 75th day.

It has been previously reported that vermicomposting modifies the original microbial community structure by allowing greater bacterial diversity, greater bacterial numbers and greater functional diversity (Vivas et al. 2009). The results indicated that the bacterial diversity did increase as the substrates were converted to vermicompost by the earthworms and then decreased in the finished product. Enhancement of microbial population correlated with the increased N and P content, which is likely contributed by the enhanced mineralization of biomass to yield nutrients, high substrate concentrations and high moisture levels. As the vermicompost begins to mature, there is a reduction in the moisture level leading to reduction in microbial population and activity (Parthasarathi and Ranganathan 1999). While we did not study the finished vermicompost during the ageing process after removal from the tanks, the microbial community

Table 1 Nutrient and chemical properties of the substrate mixture during different stages of vermicomposting of coconut leaves by *Eudrilus* sp.

Parameter	15th day	45th day	75th day	105th day
pH	5.26 ± 0.12	5.63 ± 0.31	6.40 ± 0.21	6.60 ± 0.29
Total nitrogen (%)	1.56 ± 0.16	1.32 ± 0.11	1.80 ± 0.20	1.85 ± 0.06
Total phosphorus (%)	0.14 ± 0.02	0.16 ± 0.05	0.21 ± 0.03	0.22 ± 0.03
Total potassium (%)	0.99 ± 0.05	0.36 ± 0.05	0.18 ± 0.01	0.17 ± 0.01
Phenols (ppm)	24.30 ± 3.51	21.00 ± 2.65	7.06 ± 0.81	6.03 ± 0.01
Humic acid (%)	4.23 ± 0.55	4.77 ± 0.42	9.53 ± 0.65	10.63 ± 0.84

Values presented are mean ± standard deviation from triplicate samples

Table 2 Diversity indices for the vermicompost and earthworm gut bacterial communities after normalizing for sample size

Diversity index[*]	VA_{15}	VB_{15}	VA_{45}	VB_{45}	VA_{75}	VB_{75}	VA_{105}	VB_{105}	EB_{75}
Number of sequences sampled (N)	250	250	250	250	250	250	250	250	250
Number of OTUs (S)	105	108	123	122	128	133	107	88	57
Shannon (*H*)	4.14	4.17	4.39	4.40	4.42	4.49	3.96	3.50	3.03
Simpson's reciprocal index (1/*D*)	41	44	60	60	53	61	20	13	11
$I_{\mathrm{kf}}^{\neq}$	0.228^{a}	0.223^{a}	0.206^{b}	0.214^{b}	0.255^{c}	0.257^{c}	0.246^{d}	0.249^{d}	0.223^{e}
Chao1	224	244	291	287	338	399	229	221	105
95% lci	167	179	214	212	242	278	171	151	77
95% hci	335	369	433	428	516	621	339	368	174
Richness (Ace)	436	481	457	466	670	762	459	179	201
95% lci	354	388	363	377	550	631	367	136	152
95% hci	546	606	590	590	824	930	584	260	274
Jackknife	242	265	343	363	390	942	251	305	105
95% lci	193	208	265	270	302	493	199	214	82
95% hci	291	322	421	457	479	1390	303	397	128

[*] Indices were calculated at $D = 0.03$ using a random subsample of 250 sequences per replicate and then averaged for all replicates of a sample except for the I_{kf} estimate as explained below. The acronyms lci and hci mean lower and higher confidence interval, respectively

[≠] The I_{kf} estimate of structural diversity calculated using *K*-shuff (Jangid et al. 2016). The size of libraries for these calculations was 750 for VA_{15}, VA_{45}, VB_{45}, VA_{75}, VB_{75} and VA_{105}, 500 for VB_{15}, and 250 for VB_{105} and EB_{75}. Different letters along the row indicate significant differences between communities ($p = 0.05$)

structure and biomass are likely to be reduced further (Aira et al. 2007).

Similarly, the structure of the bacterial communities changed throughout the vermicomposting process. Specifically, all bacterial phyla, except Chlorobi and Cyanobacteria, showed significant changes in abundance (Figs. 2b, S2). For instance, Acidobacteria, Actinobacteria, Chloroflexi, Gemmatimonadetes, Nitrospirae, Planctomycetes, TM7 and WS3 were significantly enriched in the 75th day samples ($p = 0.05$). In contrast, the abundance of Bacteroidetes and Proteobacteria was significantly lower in the 75th day samples. Similarly, Spirochetes showed their highest abundance in the 45th day samples, whereas Verrucomicrobia populated the first three stages fairly evenly but not the mature vermicompost (105th day samples). Firmicutes was the only phylum that consistently increased in abundance throughout the vermicomposting process. In fact, it was also the second most abundant bacterial phylum in the earthworm gut on the 75th day. Coincidently, the increase in Firmicutes abundance in the vermicompost was highest in this stage. Whether the increase in Firmicutes was inherent to the composting process and not a reflection of the bacterial addition through the microbe-rich casts from the earthworm during vermicomposting required further investigation. To ascertain this, the firmicute OTUs that were specifically shared between the vermicompost and the earthworm were examined. Notably, only one OTU was shared between the gut community with the pre-decomposition stage (15th day) and another two OTUs were shared with the mature vermicompost samples (105th day). The presence of only few shared OTUs suggested that the increase in Firmicutes was due to community succession in the vermicomposting process and not direct inoculation by the earthworms.

The total bacterial community composition was very different for the coconut leaf vermicompost than other substrates. The community composition during vermicomposting is known to differ with the substrate, the earthworm species used, and the habitat during the process (Koubova et al. 2015). Overall, Proteobacteria was the most abundant phylum followed by Bacteroidetes, Firmicutes, Acidobacteria and Actinobacteria (Fig. S3). Among the other phylogenetic groups, Chloroflexi, Gemmatimonadetes, Planctomycetes, TM7, Verrucomicrobia and WS3 were also prominent,

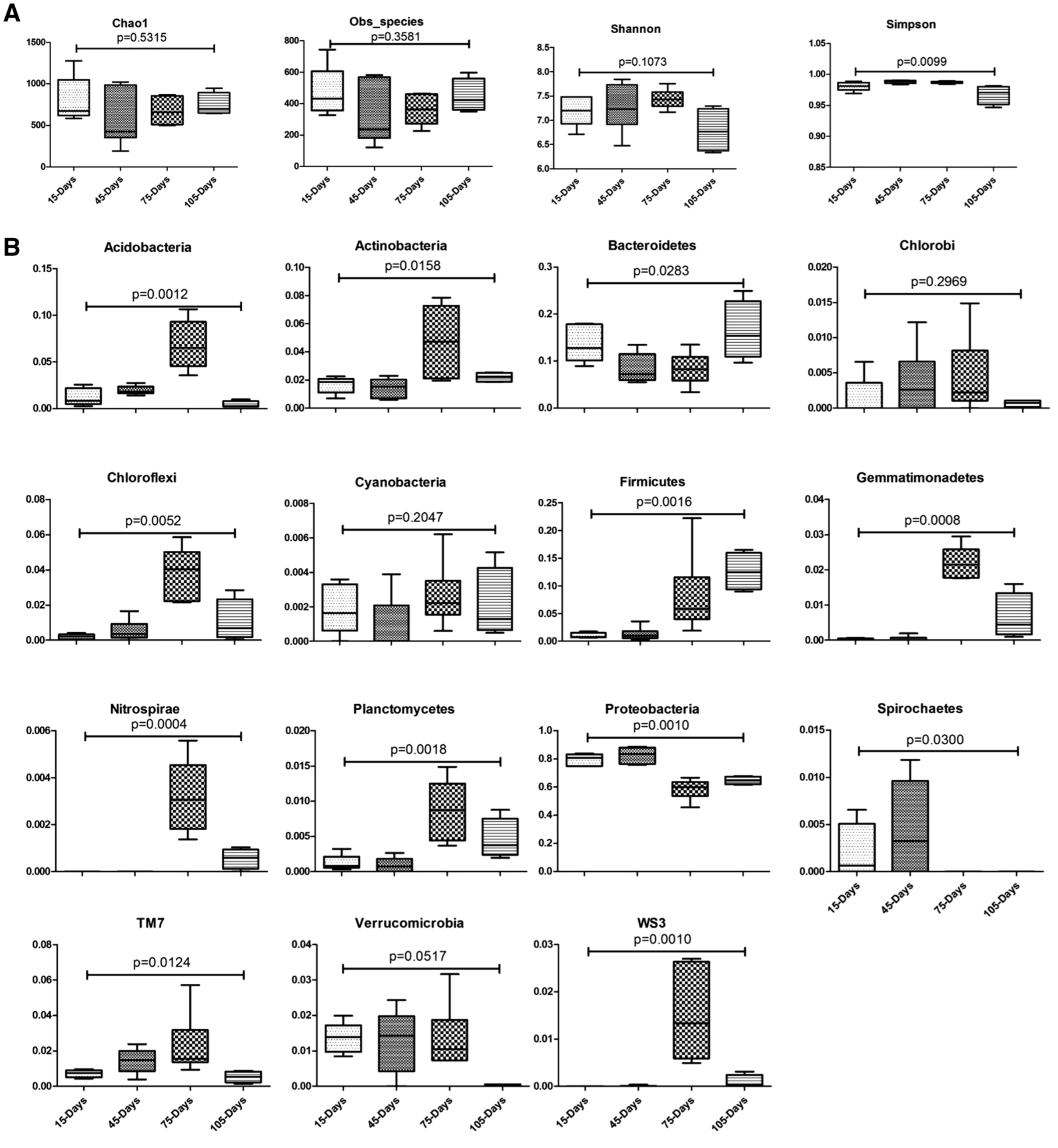

Fig. 2 Stage-wise change in diversity (**a**) and relative abundance of major phyla (**b**) during coconut-leaf vermicomposting. For **a**, values on *y-axis* indicate the mean value of the diversity index calculated from all replicates (five for 15th day, six each for 45th and 75th day, and four for 105th day) for a particular sampling time point. For **b**, values on the *y-axis* represent mean relative abundance of the phyla present in all the replicates for a particular sampling time point. *P-values* indicate statistical significance calculated using the Kruskal–Wallis test across the four stages

whereas Chlorobi, Cyanobacteria and Nitrospirae were the least abundant. In comparison, culturing studies conducted earlier on mature coconut leaf vermicompost and vermicasts led to the isolation of only a very small fraction of genera of bacteria—*Bacillus, Xanthomonas, Micrococcus, Arthrobacter, Pseudomonas, Flavobacterium, Alcaligenes*; and actinomycetes—*Actinomyces*, *Streptomyces*, *Micromonospora*, *Nocardia* and *Thermoactinomyces* (Thomas et al. 2012). Although other high throughput sequencing studies of vermicomposting were not available for comparison, single-strand conformation polymorphism (SSCP) was used to demonstrate changes in the microbial communities of vermicompost of agricultural crop residues processed by *Eisenia foetida* (Fracchia et al. 2006). The agricultural vermicompost was dominated by Chloroflexi, Acidobacteria, Bacteroidetes and Gemmatimonadetes. While the Chloroflexi and Gemmatimonadetes peaked on the 75th day in our studies, they never dominated and were depleted in the mature coconut leaf vermicompost. It is therefore very likely that the coconut leaf vermicompost possessed a very different microbial community.

The succession of dominant bacteria was also reflected in the overall change in community composition during vermicomposting. Based on the analysis of cross-K-functions using *K*-shuff (Fig. 3) and both weighted and unweighted UniFrac (Fig. S4), the bacterial communities showed a stage-dependent clustering. Such stage-wise clustering was not unexpected given the variation in the chemical and nutrient properties as well as changes in the nutrient availability within the vermicomposting microenvironment. Further, the earthworm gut bacterial community was very different from that of all four vermicompost stages, suggesting that the earthworm gut communities were unique.

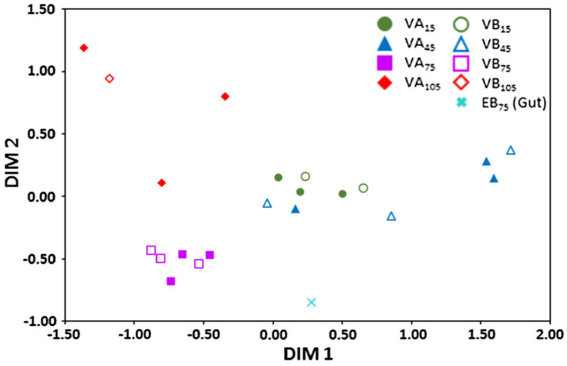

Fig. 3 MDS plot of the compositional parameter C_{kf} for bacterial communities from coconut-leaf vermicomposting and earthworm gut (EB$_{75}$). Labels prefixed with VA and VB represent replicate samples collected from two separate tanks at the specified time points

Specific associations of the most abundant OTUs

The pyrosequencing data was examined for evidence of distinct stage-specific populations during vermicomposting process and in the earthworm gut to improve our understanding of the microbial dynamics. The stage-specific microbiome was defined as those OTUs that were specifically associated ($p = 0.05$) with only one or two different stages during the vermicomposting process. A total of 351 such OTUs were identified (Tables 3, S3). Of these, 116 OTUs were associated with a single stage: 21 in 15th day, five in 45th day, 34 in 75th day and 56 in 105th day. The remaining 235 OTUs were associated with two different stages of vermicomposting (Table S3). An in-depth analysis of the taxonomy of all 351 OTUs indicated that the largest group were Proteobacteria followed by Firmicutes. Among the Proteobacteria, Alphaproteobacteria was the single largest class followed by Gammaproteobacteria, Betaproteobacteria and Deltaproteobacteria. Further, the most abundant of these stage-specific OTUs, #1129906 was affiliated with the genus *Wautersiella* (a Bacteroidetes) and was associated with 105th day samples only. It is interesting to note that this bacterium has been found in abundance in the rumen of cattle as a biofilm community (Petri et al. 2013) and could have been introduced into this vermicomposting environment from the cow dung added during the pre-decomposition stage. *Wautersiella falsenii* has also been isolated from diesel-contaminated food waste composts (Lin et al. 2011) and clinical samples (Kämpfer et al. 2006). It is interesting that this genus appeared in abundance in the last stage of coconut leaf vermicomposting process whereas cow dung was added during the first stage. Although we could not detect *Wautersiella* in earthworm samples, the possibility of its multiplication and passage through the worm's gut could not be ruled out (Gopal et al. 2006). Similar to *Wautersiella*, the second most abundant stage-specific OTU, a *Sphingobacterium* sp. (OTU#436314), was also specifically abundant in the 105th day samples. Four related species have been isolated from compost samples (Ten et al. 2006; Kim et al. 2006; Yoo et al. 2007; Yabe et al. 2013), and the frequent isolation of sphingobacterial strains from compost sites is an indication that this group is likely to be an important contributor to many types of the composting. Members of this 'stage-specific microbiome' may be

Table 3 Five most abundant OTUs detected within each stage of coconut leaf vermicomposting by *Eudrilus* sp.

OTU Number	15th day	45th day	75th day	105th day	Taxonomy[a]
5860	9.60	0	0	0	*Hyphomonas*
991740	8.20	0	0	0	*Sediminibacterium*
570755	5.20	0	0	0	*Pedobacter*
543389	5.00	0	0	0	*Pseudomonas*
825033	2.00	0	0	0	*Erwinia*
4383097	0	1.67	0	0	*Flavobacterium*
2954724	0	1.33	0	0	Acetobacteraceae
687946	0	0.83	0	0	Alphaproteobacteria
3016490	0	0.50	0	0	OM60
539430	0	0.50	0	0	*Opitutus*
2363675	0	0	6.17	0	Sphingobacteriales
361902	0	0	6.00	0	Sphingobacteriales
4389974	0	0	5.00	0	Acidobacteria-6
258814	0	0	4.33	0	Myxococcales
538089	0	0	4.33	0	MND1
1129906	0	0	0	275.50	*Wautersiella*
436314	0	0	0	124.50	*Sphingobacterium*
526024	0	0	0	63.50	*Pseudomonas*
167215	0	0	0	39.50	Clostridiaceae
242503	0	0	0	18.00	*Alkaliphilus*

Values presented are the mean of actual abundance in terms of number of sequences present in all replicates (five for 15th day, six each for 45th and 75th day, and four for 105th day) and statistically significant by the Kruskal–Wallis test (p = 0.05). Zero indicates OTUs that were completely undetected in any of the replicates for that time point

[a] The most specific taxonomic level assigned to the OTU

explored and manipulated for further improvement in the vermicomposting process.

Similar to the stage-specific microbiome analyses, we looked at the overall distribution of OTUs across all stages and identified varying distribution patterns (Fig. S5). For instance, the most abundant OTU (#564678) across all stages, a *Pseudomonas* sp. decreased in abundance from the initial high levels on 15th day (mean abundance from six replicates = 26.6) to the 75th day (mean = 5.8) but then increased 10-fold in the mature vermicompost (mean = 265.25). Certain other OTUs showed an opposite trend. For instance, the fourth most abundant OTU (#309900), an *Acidovorax delafieldii,* was highly abundant at the beginning (15th day mean = 153.4) but then decreased consistently during the intermittent two stages (45th day mean = 43.3, 75th day mean = 0.17) before the abundance dropped below the level of detection in the mature vermicompost on the 105th day. Five strains of this bacterial species were originally isolated from soil as *Pseudomonas delafieldii* by Delafield et al. (1965) on culture media with poly-β-hydroxybutyrate as the sole carbon source. Pseudomonads are known to degrade a variety of complex substrates, and their high abundance in the vermicompost samples was not surprising.

Analysis of the functional capacity of bacterial communities during vermicomposting

To understand the functional implications of these shifts during vermicomposting, the biochemical potential of the associated OTUs were predicted with an imputed metagenomics approach (Fig. 4). A total of 117 metabolic functions that grouped into 12 categories were identified. While OTUs with specialized functions were found specifically associated with certain stages of the process, the diversity of functional pathways was predicted to be higher during the latter stages of vermicomposting. For instance, OTUs specialized in enzymes and pathways of lipid metabolism (ether lipid metabolism, and synthesis and degradation of ketone bodies) were specifically predicted to be more abundant at the beginning of vermicomposting. Whereas, those having pathways for the metabolism of terpenoids and polyketides, metabolism of glutathione, and β-alanine and geraniol degradation were predicted to be enriched in the mid-

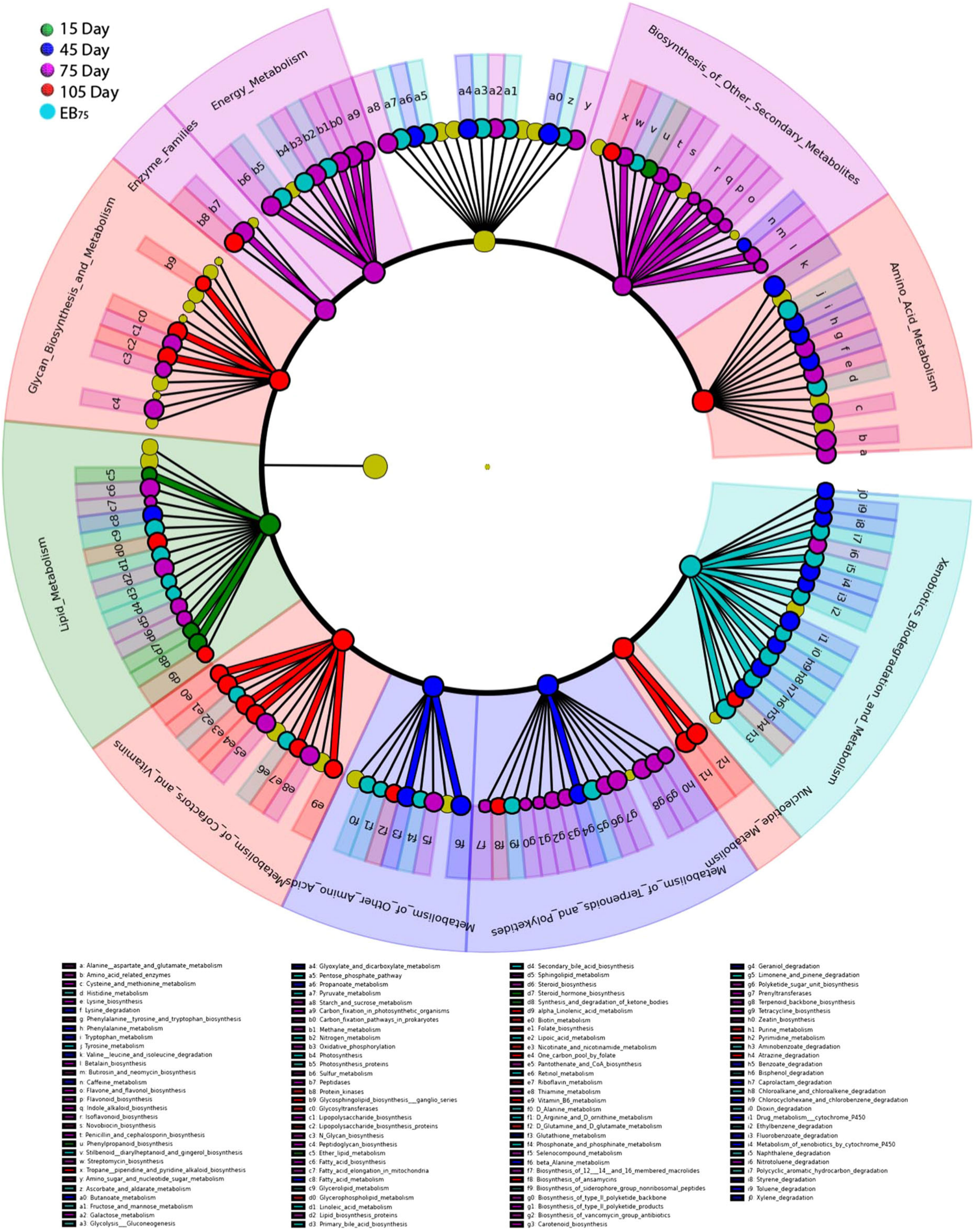

Fig. 4 Graphical presentation of imputed metagenome in vermicompost and earthworm gut samples. Cladogram showing differential abundance of metabolic functions of bacterial origin

stage on the 45th day. On the 75th day, OTUs possessing enzymes for sulfur metabolism and biosynthesis of secondary metabolites along with energy metabolism were specifically predicted to be abundant. Further analysis into the secondary metabolites predicted the presence of pathways involved in biosynthesis of antibiotics (butirosin, neomycin, novobiocin, penicillin and streptomycin), indole alkaloid and pigments (betalain, flavone, flavonoid). The presence of antibiotic production pathways suggested the likely abundance of certain actinobacteria during this stage (Fig. 4). Indeed, *Streptomyces* spp. were highly abundant in the 75th day vermicompost samples. Lastly, enzymes involved in the metabolism of nucleotides, cofactors and vitamins, and glycan biosynthesis were predicted to be highly enriched in the mature vermicompost on the 105th day.

Of special interest was the presence of OTUs with lignin degradation capacity, which persisted throughout the vermicomposting process. However, the database for bacterial lignin degradation is very limited and only a few bacterial taxa, such as *Acinetobacter*, *Bacillus*, *Pseudomonas*, *Rhodococcus*, *Sphingomonas* and *Streptomyces* are known to harbor these enzymes and pathways (Bugg et al. 2011; de Gonzalo et al. 2016). In addition, very limited information is available in the KEGG database with the only known lignin degrading proteins belonging to *Agrobacterium* spp., *Rhizobium etli* and *Sorangium cellulosum*. OTUs affiliated with these three species were abundant in the coconut-leaf vermicompost samples too. Specifically, *Rhizobium* was more abundant in 15th day samples at the beginning, *Agrobacterium* in the mid-stages in 45th day samples, and *Sorangium* was detected at low abundance near the completion of vermicomposting in 75th and 105th day samples. Presumably, the limited number of lignin-degrading bacteria reflects the incompleteness of the reference database. Regardless, it was evident that the potential for lignin degradation persisted throughout the vermicomposting process.

Of further interest was the presence of plant growth promotion properties in the mature vermicompost, which is a major reason for its popularity with farmers. Three functions: carbon fixation in photosynthetic organisms, carbon fixation pathways in prokaryotes and indole alkaloid biosynthesis (all in the 75th day sample) were quite interesting from this perspective. The higher abundance of carbon fixation in photosynthetic organisms was very likely contributed by Cyanobacteria, which were slightly more abundant in the 75th day samples. Similarly, the pathway for indole alkaloid biosynthesis was predicted to peak on 75th day. Plant growth hormones, such as 1-naphthaleneacetic acid and indole-3-acetic acid inhibit the accumulation of alkaloids by downregulating the tryptophan decarboxylase gene involved in the indole alkaloid biosynthesis pathway (Zhu et al. 2015; Pasquali et al. 1992). Concurrently, the 45th day samples likely possessed an increased degradative capacity for geraniol, which is involved in alkaloid accumulation (Papon et al. 2005). Both functional attributes would lower the levels of alkaloids in the mature vermicompost, thereby suggesting an indirect mechanism for enhancement of plant growth by the vermicompost microbiome. Based on culture-dependent analysis of bacterial communities during coconut leaf vermicomposting by *Eudrilus* sp., Gopal et al. (2009) reported an increase in the population of plant-beneficial microbial communities. Similarly, combined application of coconut leaf vermicompost and biofertilizer also increased the chlorophyll index in the leaves of coconut palms as compared to chemical fertilizer application (Hebbar et al. 2016). These findings further support the use of vermicompost for improving plant productivity.

Linking the bacterial communities of substrate and earthworm gut

One of the main objectives of this study was to examine the role of the earthworm microbial community on vermicomposting and whether the earthworm community could serve as the inoculum for the major decomposers of the vermicompost microbial community. For the latter to be true, the OTUs present during the early or middle vermicomposting stages must also be shared with those in the earthworm guts. Alternatively, the earthworms could be important processors of the substrate to make it more palatable to the endogenous microbial community, and there would be no direct link between the OTUs in the earthworms and vermicompost. While it was difficult to conclude with certainty, all analyses suggested that the earthworm gut bacterial community was likely to be unique to the host. In comparison with the vermicompost bacterial community, the earthworm gut bacterial community (EB_{75}) showed consistently lower diversity (Tables 2, S2; Fig. S1) and significantly different composition at the phylum level (Figs. S2, S3) as well as for the overall community (Figs. 3, S4). Further support for this conclusion was garnered from the distribution of the most abundant OTUs in the earthworm gut. Of the five most abundant OTUs at each stage of the vermicomposting process, only a single OTU was shared between the earthworm gut (EB_{75}) and the vermicompost samples collected on 75th day. This OTU was a firmicute and closely related to *Bacillus* spp. Coincidently, Firmicutes are known to be common members of both the gut and vermicompost communities. All other OTUs regardless of the stage were absent from the earthworm gut sample. Functionally too, the earthworm gut bacterial community was unique as it was predicted to be highly enriched for the degradation of benzoics and aromatics (aminobenzoate, bisphenol, chloroalkane and chloroalkene, dioxin, naphthalene, polycyclic aromatic hydrocarbons) (Fig. 4). These results collectively suggested that the bacterial community in the earthworm gut was significantly different from that in vermicompost and possessed unique functional ability.

There is conflicting evidence regarding the uniqueness of the earthworm gut community and its role in the composting process. Based on qPCR assays of bacterial communities in earthworms, Rudi et al. (2009) showed that although feeding led to stabilization of the gut microbiota, it was still very distinct from that of the bedding and feed. In contrast, Aira and Dominguez (2011) studied the indirect effects of vermicomposting by using worm-worked pig manure as an inoculum for composting fresh pig manure and compared these with direct effects when composting was carried out using three different earthworms (i.e., *E. andrei*, *E. foetida* and *E. eugeniae*). While the inoculation of worm-worked pig manure significantly increased microbial biomass and enzyme activities, these effects were lower than when earthworms were present and dependent on the earthworm species. It is also known that enzyme activities, except those associated with humic compounds, tend to decrease with time during vermicomposting (Nechitaylo et al. 2010). Likewise, it was not the parent material, but the earthworm species that largely determined the structure of microbial communities in the finished

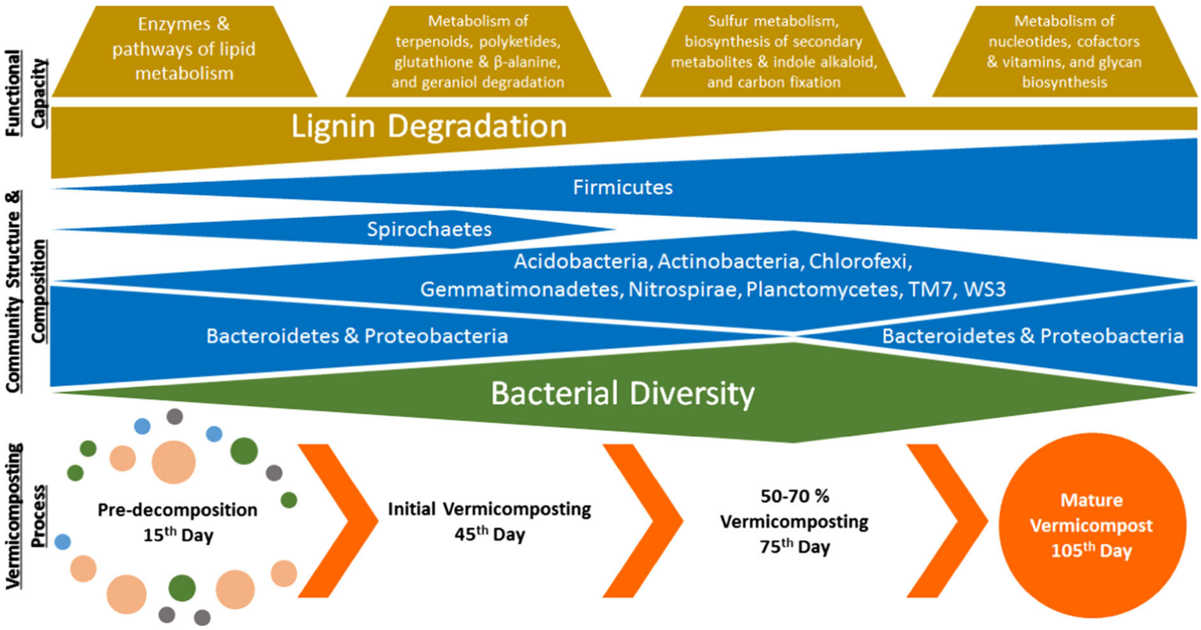

Fig. 5 Change in bacterial communities associated with each stage of coconut leaf vermicomposting process. Change in the measured property mentioned on the *left* is indicated by the change in height of horizontal boxes from *left to right* across the figure. For instance, the bacterial diversity peaked in the 75th day samples. Similarly, the increase in abundance of Firmicutes was consistent throughout the vermicomposting process. Further, OTUs possessing lignin degradation pathways were most abundant in the beginning followed by a drop which then stabilized in the 75th and 105th day samples

vermicompost (Lores et al. (2006). Similarly, pyrosequencing revealed a more complex bacterial community structure for earthworm-treated substrate as compared to a control group, suggesting that earthworms modified the structure of microbial communities during vermicomposting (Chen et al. 2015). Earthworms are known to affect the nutrient dynamics during the process of vermicomposting (Dominguez and Gomez-Brandon 2013). They aid in the rapid decomposition and mineralization of the organic matter in sewage sludge vermicomposting, enhance total potassium, calcium and iron content, as well as modify phosphorus and zinc to a more utilizable form. These results suggest that the earthworm gut community is not only responsible for increasing the quality of the vermicompost but also aids in increasing the efficiency of the process. In the present study, several mechanisms are likely in play during the vermicomposting process. Further, the association between the microbial communities during vermicomposting and the earthworm gut is far more complex and requires a thorough and systematic investigation.

Conclusions

This study revealed the succession of bacterial communities and their activities during the vermicomposting of coconut leaves by *Eudrilus* sp. (Fig. 5). Communities peaked in diversity during the mid-vermicomposting stage (75th day) of the process. This increase in diversity was associated with a concomitant increase in the abundance of a number of bacterial phyla and a decrease in the abundance of Bacteroidetes and Proteobacteria on the 75th day. Firmicutes showed a consistent increase in relative abundance throughout the vermicomposting process. The changes in the relative abundances during vermicomposting were sufficiently distinct that communities clustered separately in a stage-dependent order. The succession of bacterial activities was also clearly evident with the pathways of lipid metabolism and to some extent lignin degradation, which were more abundant at the beginning, followed by those involved in the biosynthesis of secondary metabolites and plant beneficial properties, such as carbon fixation and indole alkaloid biosynthesis during the later stages of vermicomposting. The structure, composition and activities of the bacterial communities in the earthworm gut on the 75th day were unique and differed significantly from any of the vermicomposting stages. The stage-specific association(s) of some of the most abundant OTUs yielded important clues about the members, which could serve as potential targets for further improvements in the vermicomposting process.

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Conflict of interest The authors declared that they have no conflict of interest.

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