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Metagenomic approach unravelling bacterial diversity in combined composting and vermicomposting technology of agricultural wastes

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Abstract. Agricultural wastes and their valorization are at the heart of the EU policies related to the circular bioeconomy. Conversion of those wastes to products could be made through composting followed by vermicomposting. As the effectiveness mostly depends on the microorganisms involved, we aimed to identify the prokaryotic microbiome associated with four composting phases and one vermicomposting phase of wheat straw and cow manure composting. We used 16S rDNA PCR amplicon evaluation with the Illumina metagenomic technique generating a total of 653,057 sequences reads from the samples. Temperature had major role in the composting bacteriome changes influencing positively species abundance, Shannon and Simpson indices, and negatively Ace and Chao1. A reduction of C:N ratio from 25.94 to 14.24 and of pH and EC from 8.63 to 7.8 and from 2.26 $mS.cm^{-1}$ to 1.7 $mS.cm^{-1}$, respectively, were observed. Phylum *Firmicutes* (62%) and *Actinobacteria* (14%) prevailed in the source material (SM), while *Proteobacteria* (51%) and *Bacteroidetes* (8%) dominated in the first mesophilic phase (MP). Similarly to the SM, the thermophilic prokaryotes (TP) were represented by *Firmicutes* (54%) and *Actinobacteria* (9%), but also by *Proteobacteria* (6%). Principal coordinates analysis (PCoA) showed a significant weight of the total variation of bacterial taxa (PC1-68.16% and PC2-23.46%. Thus PCoA grouped together SM and TP, by one site, and both mesophilic phases and the vermicompost (COMP).

Key words: agricultural wastes, composting, vermicomposting, phases, prokaryotic microbiome, bacterial diversity, metagenomics, NGS.

Introduction

Composting is one of the most widely used technologies for agricultural waste valorization. It is an aerobic biological process of degradation of organic compounds by microorganisms (Haug, 1993; Vargas-Garcia et al., 2010). During composting, the organic residues are converted into organic compounds beneficial for plant nutrition, improving soil structure and fertility, organic content, water holding capacity, and so on. The process could be conditionally divided into phases characterized by different abiotic and biotic characteristics. Besides the differences in temperature, pH, O_2 and water content, the phases vary widely by the microorganisms involved and their processes (Shilev et al., 2007; Partanen et al., 2010; Scotti et al., 2016). The source material is essential and partially influences the microbiome structure (Scotti et al., 2016). In some cases, the resulting compost is offered to earthworms as additional vermicomposting treatment that reduces the overall time for composting, accelerates the lignocellulosic degradation and improves the final product quality

according to Singh et al. (2002). In recent years, composting and vermicomposting technologies changed and were optimized to meet society's new needs and environmental protection (Zhou et al., 2022).

Diverse culture-dependent methods have been applied to uncover the microbial diversity of composting (Steger et al., 2007; Angelova et al., 2016;). Recently, investigators have created various molecular techniques to collect information concerning the microbial community composition of the compost. Some methods are amplified rDNA restriction analysis, DNA hybridization or investigative microarrays (Franke-Whittle, 2009; Wu et al., 2017; Lui et al., 2022). Shotgun metagenomics has become a valuable method to identify the microbiome composition in compost and soil samples (Hayat et al., 2013; Wang et al., 2015).

Among the very diverse analyses applied to reveal the microbial composition and diversity of composting, the phylum *Actinobacteriota, Proteobacteria, Acidobacteriota, Firmicutes,* and *Bacillota* are most often reported (Guo et al., 2007; Martins, 2013; Chopkova et al., 2023). As the initial mesophilic phase often possesses relatively low pH, the observed microbiota belonged to the phylum *Firmicutes*, order *Lactobacillales* (Ishii & Takii, 2003; Partanen et al., 2010; Martins et al., 2013). In a previous study, we found higher abundance in the mesophilic phase of representatives of class *Alphaproteobacteria, Actinobacteria,* and *Gammaproteobacteria,* among others. In the thermophilic phase, *Bacilliota* and *Actinobacteriota* are mostly reported (Guo et al., 2007; Chopkova et al., 2023). At the genus level, *Bacillus, Thermus, Geobacillus* were found as the most abundant thermophilic bacteria (Zhang et al., 2002; Poli et al., 2011; Finore et al., 2023). However, we still have limited knowledge of microbial community structures in the composting process due to microbial complexity and the limitation of detection methods (Wang et al., 2018a). Previous studies of microbial community dynamics in different composting systems, such as in cow dung and wood chips (Wang et al., 2018b), corn cobs and fresh cow dung (Zhang et al., 2021), plant waste and cow dung (Varma et al., 2018), also reported insufficient scientific information and needs of further investigations. Therefore, it is necessary to assess the sequence of the composting microbial community using high-throughput sequencing technology.

The abovementioned literature showed the importance of combining different tools in investigating the succession changes in the composting process. The information on the bacteria diversity and abundance in composting phases is not sufficiently well examined, especially from the point of view of the extreme variety of agricultural waste and composting systems. In our study, we investigated the changes in the prokaryotic microbiome during the composting/vermicomposting of agricultural waste from the source materials to the final vermicompost.

Materials and Methods

Experimental design and sampling

The composting experiment was carried out at the site of a company producing compost in Kalekovets village, Plovdiv district, South Bulgaria. Biodegradable farm wastes were mixed to obtain quality organic fertilizer compost and demonstrate agriculture's circularity. Four tones of cow manure and 1.5 tons of wheat straw were thoroughly mixed, getting C:N ratio of 25.94 and forming a pile with dimensions 3 m width, 5 m long, and 1.2-1.3 m height. The process started in late April 2022 on the concrete ground turning the biomass mechanically 1-2 times per week and maintaining the water content 50-55% of water holding capacity (WHC). Three samples were taken from each target: the source materials, first mesophilic phase, thermophilic phase, second mesophilic phase and vermicompost for posterior analyses. The material was finely chopped in the laboratory. After five months, the resulting compost was supplied with earthworms *Eisenia fetida* (Sav.) and *Lumbricus rubellus* (Hoff.) at a concentration of 30000 un./m^3. The vermicomposting process was conducted for two months, after which the final vermicompost was sieved and prepared for analysis.

Physicochemical properties

To prepare the samples for analysis, they were first very well milled. Ten grams of compost samples were added to 50 ml of distilled water (1:5, w/v) and shaken for 60 min. (Petkova et al., 2020; Petkova & Shilev, 2023). After 30 min. of sedimentation the pH and electrical conductivity (EC) were measured using WTW pH-EC meter (Rhoades, 1996; Thomas, 1996). Total nitrogen content was assessed through the Kjeldahl method. The ammonia (NH_4-N) and nitrate (NO_3-N) were extracted using 2 M KCl (1:10, w/v). After centrifugation and filtration the ammonia was determined by NaOH distillation and titration with H_2SO_4. Nitrate concentration was calculated as the difference between the values of Zn-$FeSO_4$ and NH_4-N according to Bao (1981). The samples were carbonized in a muffle furnace at 550°C for six hours to measure the organic C as was published by Angelova et al. (2019).

Basal and substrate-induced respirations (BR, SIR) were carried out by the method described by Alef (1995). It consisted of placing 50 g of fresh soil in a container with a small beaker with 20 ml 0.05 M NaON. After the desired exposure time the free NaOH is titrated with 0.05 M HCl until it changes from red to colorless. The heavy metal concentrations were determined using wet mineralization method described by Lozano-Rodrigues et al. (1995).

Isolation and purification of DNA from compost and metagenomic sequencing

DNA extraction and purification were obtained using the DNA extraction method described by Caporaso et al. (2011) from five different compost phases: source material (SM), first mesophilic stage (MP), thermophilic stage (TP), second mesophilic stage (MP2), and vermicompost (COMP). The amplification of PCR products for the 16S region with 16S V3-V4 primers (CCTAYGGGRBGCASCAG, GGACTACNNGGGTATCTAAT) was successful for the all five samples (Caporaso et al., 2012). The amplicon was sequenced on an Illumina double-end platform to produce 250 bp raw double-end reads (Raw PE) and then pooled and preprocessed to obtain clean tags (Krstić Tomić et al., 2023). Chimeric sequences in the pure markers were detected and removed to get the effective markers that could be used for subsequent analysis, as published by Chopkova et al. (2023) and Krstić Tomić et al. (2023). Metagenomic sequencing was done at Novogene (Cambridge, UK). Library preparation was done with the Nextera DNA Flex kit (Illumina) following a standard procedure. The exact amount of PCR products from each sample were pooled, A-tailed and further ligated with Illumina adapters and then pooled and preprocessed to obtain clean tags. The raw data are available at NCBI plat-form under submission number SUB14004006 of 28.11.2023 for BioProject ID PRJNA1045869 with following BioSample accessions: SAMN38457370, SAMN38457371, SAMN38457372, SAMN38457373, SAMN38457374.

Data were processed using QIIME software, v.1.9.1 (http://qiime.org/) (Caporaso et al., 2010). The first phase of 16S rRNA gene analysis involves quality control of sequences to exclude from analysis those less than 200 nucleotides in length, with a quality score of less than 25, with misread primer sequences and multiplex identifiers, extensive homopolymeric repeats (more than 8 nucleotides) and unidentified nucleotides. Chimeric sequences in clean tags were removed to obtain efficient tags used for subsequent analysis (Haas et al., 2011; Martin, 2011; Edgar, 2013).

Operational taxonomy units (OTUs) were selected at greater than 97% similarity. Scores of Ace and Chao indices and Shannon and Simpson diversity indices were calculated using the Mothur program (Abell & Bowman, 2005; Tupak et al., 2017;). Alpha diversity metrics summarize the structure of an ecological community by measuring the number of taxonomic groups along with group abundance, as published by Willis (2019). Alpha diversity was analyzed using six indices, including observed species, ACE, Shannon, Simpson, Chao1, and good cover, and calculated using QIIME (Version 1.9.1, http://qiime.org/1.9.1/) and displayed with R software (Version 2.15.3) (Caporaso et al., 2010). The heat map based on weighted Unifrac and unweighted Unifrac distances

was analyzed with R software (Version 2.15.3). The same R software was used to find the differences in dominant taxa among the three groups of samples at each taxonomic rank. The top 10 taxa with the mean abundance of the three groups of samples at each taxonomic rank were selected to generate a triple plot. OTU comparisons were performed using the Venn diagram package.

The beta-diversity study assessed differences in diversity levels between two soil samples. Beta diversity results were calculated using QIIME software (Version 1.9.1). The results are presented as the distance between samples and include both the weighted and unweighted distance (Lozupone et al., 2005; Lozupone, 2006). It is a suitable method for analyzing the sparse soil microbiomes. All studied physicochemical factors during the composting process and microbial diversity indices were correlated one wise with each other using Pearson correlations to identify relationships between them ($p<0.05$).

All significance tests were two-tailed, and values of $p<0.05$ were considered statistically significant. In our 16S information analysis process, the input file of Principal coordinates analysis (PCoA) is the beta-diversity distance matrix, which is the difference value matrix composed of the OTU abundances of two samples.

Results

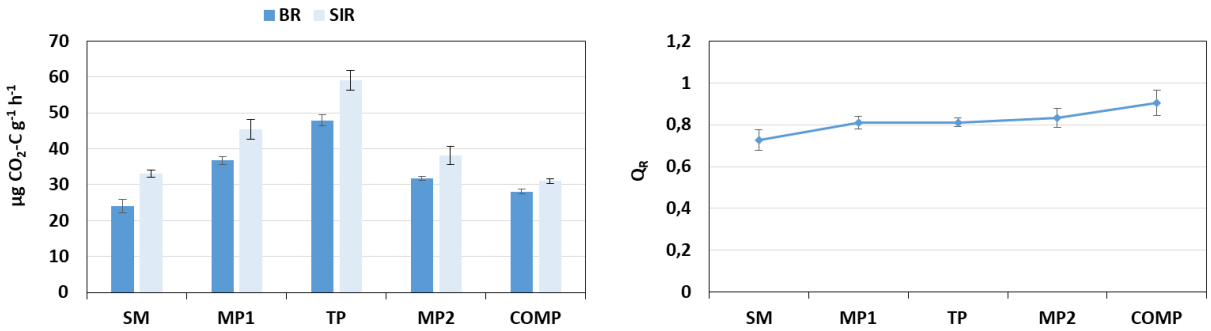
Physicochemical properties of composting process and respiration

The chemical properties of different phases of the composting process are presented in Table 1. The change in the content of chemical elements is characteristic of the composting process. Total organic carbon (TOC) decreases as the composting progresses due to microbial communities' conversion of organic compounds, with vermicompost reducing TOC by 50%. The highest significance was found between source materials and final vermicompost. At the same time, total N decreased by 9.3%. Thus, the critical C:N ratio is reduced from the initial 25.94, often considered as optimal (Zhang et al., 2015) to the final 14.24 in vermicompost. According to the Bulgarian national legislation, to be considered as a compost, the final product must have a ratio below 15 (MOEW, 2017). In a similar study, Wei et al. (2018) found a C:N ratio of 22.57 in the final product starting from 31.28. This parameter affects the microbiome community compositions in different composting phases (Eiland et al., 2001). After the initial increase in total P concentration, the values were reduced with 32.3% from the first mesophilic phase till the final vermicompost. The increase in concentration was attributed to the loss of water content and concentration of organic matter. Similar findings were reached by other researchers (Wei et al., 2018). There was also found a decrease in the concentration of Ca, Mg and Fe. The EC and pH also decreased during the process. Thus the final value was 7.8 for pH (9.6% of decrease), while EC lowered by 24.8% to 1.7 $mS.cm^{-1}$. The differences between the phases were significant except between the SM and MP. The trend of both parameters showed a stabilization of the composting material.

Lower microbial activity expressed as basal respiration was found in agricultural wastes before composting (Fig. 1). The values of this parameter have increased in MP and TP followed by a decrease. Lower BR was found in the COMP. Moreover, the SIR is higher than the values of BR in all samples and phases, between 10-20%. The metabolic quotient estimates microbial respiration per active microbial biomass C. In our study, it ranged between 0.73 and 0.9, being lower at SM and higher in the COMP. It is known that the nitrogen loss during composting is mainly due to the microbial activity (Tong et al., 2019). In addition, the carbon loss follows the N ones and may reach more than 80% especially due to the CO_2 emissions (Guo et al., 2012). From this point of view, the respiration is an indicator of compost stability (Sánchez-García et al., 2015). Qu et al. (2020) found similar trends of respiration when studied composting of diverse sources. Higher respiration rates were found during thermophilic phase and lower in final product in all treatments.

Table 1. Chemical parameters of compost phases. The results represent the mean and the S.E. (n=3). Different letters show significant differences among composting phases according Student's t-test ($p<0.05$).

Parameters	SM	MP	TP	MP2	COMP
TOC (%)	36.31±1.6a	29.47±1.0b	25.11±1.3b	22.74±1.8b	18.09±1.5c
Total N (%)	1.40±0.16a	1.55±0.23a	1.40±0.16a	1.51±0.21a	1.27±0.18b
C/N ratio	25.94a	19.02b	17.94b	15.06c	14.24c
Total P (%)	1.07±0.11a	1.61±0.05a	1.48±0.09b	1.37±0.21b	1.09±0.09c
Ca (%)	3.28±0.11a	3.89±0.19b	3.31±0.20a	3.47±0.16c	3.12±0.17d
Mg (%)	0.87±0.07a	0.89±0.07a	0.84±0.08a	0.78±0.09b	0.74±0.08b
Fe (%)	0.76±0.08a	0.73±0.09a	0.76±0.05a	0.58±0.07b	0.53±0.05b
pH	8.63±0.02a	8.57±0.01a	8.45±0.01b	8.2±0.01c	7.8±0.01d
EC (mS.cm^{-1})	2.26±0.01a	2.20±0.0a	1.94±0.0b	1.8±0.01c	1.7±0.01d
Temperature	25	45	66.3	47	30



(a) (b)

Fig. 1. Basal respiration and induced respiration (a), and metabolic quotient (b). The results represent the mean and the S.E. (n=3).

Composting and vermicomposting bacterial diversity

After quality filtering, 653,057 (average 130,611 per sample) sequence reads were clustered into OTUs (average 3290 per sample). The remaining sequences (unique tags: 8731) were not related to any known bacterial sequences in the public database. These results indicate that the sequencing depth is sufficient to capture a respectable number of observed bacterial species. The mature straw and manure compost had the highest number of species observed while having advanced bacterial diversity compared to the other sites.

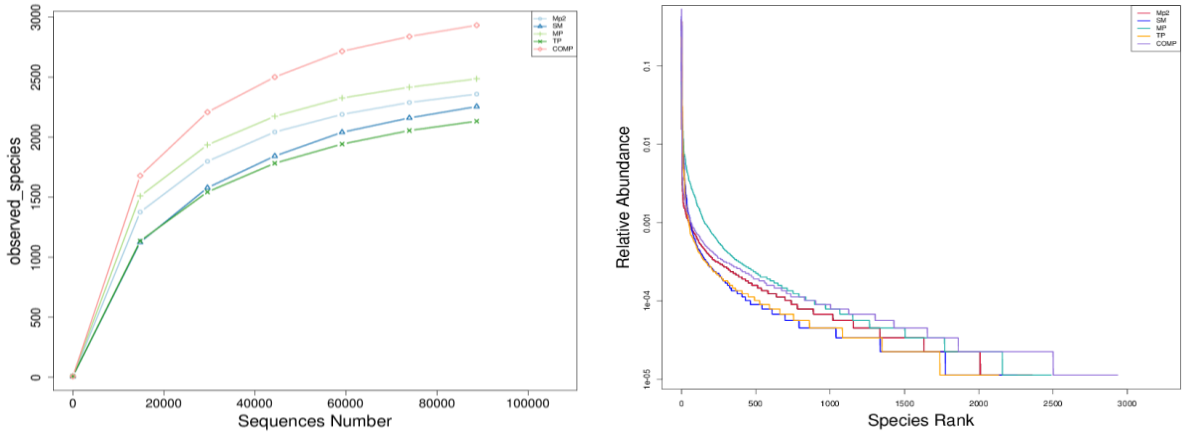
Rarefaction curves were calculated for all five samples (Fig. 2a), plateaued at approximately 88719 sequences. The more significant number of observed species corresponds to greater bacterial diversity. Rarefaction curves can directly reflect the rationality of the sequencing data volume and indirectly reflect the richness of the microbial community in the samples. If the curve is steep, it means that many of the species remain to be discovered. If the curve becomes flatter, a reliable number of samples have been taken, meaning that only the rare species remain to be sampled. Five different compost phases are shown in Figure 1, and the vermicompost (COMP) showed higher richness. Generally, the observed species number decreased in the following order: MP, MP2, SM, and TP. The bacteriome in the thermophilic phase presented a lower number of species.

The abundance curves in Figure 2b are used to sort the characteristic sequences in the samples by relative abundance (or the number of sequences included) from largest to smallest to obtain the corresponding sort number. Similar to the analysis of prokaryotic

community richness, the relative abundance of species in the mature compost is measured as the slope of the rank abundance curve (RAC), where steeper slopes reflect greater dominance. Conversely, flatter slopes in SM and TP indicated greater uniformity.

Table 2. Summarization of the tags and OTUs number obtained at each composting phase (SM – source materials; MP – first mesophilic phase; TP – thermophilic phase; MP2 – second mesophilic phase; COMP – final vermicompost).

Sample Name	Raw PE	Combined	Qualified	Nochime	Base(nt)	AvgLent	GC, %	Q20, %	Q30, %	Effective%
SM	131803	130558	127938	115282	48679207	422.26	56.11	97.83	93.37	87.47
MP	136211	135292	133044	115763	48400394	418.10	54.37	98.22	94.13	84.99
TP	134734	133228	130724	115191	48384082	420.03	56.31	97.82	93.29	85.50
MP2	129971	128895	126592	107591	45623413	424.04	54.82	98.01	93.75	82.78
COMP	120338	119438	117451	96789	40893845	422.51	54.94	98.16	94.03	80.43



(a) (b)

Fig. 2. Rarefaction curves based on the number of observed species (a) and species relative abundance curves (b).

In our study, the structure of composting bacteriome was significantly changed during the biological process. *Firmicutes* dominated the bacteriome of SM (62%) together with *Actinobacteria* (14%) (Fig. 3a). The representatives of this phylum strongly decreased in the MP, where *Proteobacteria* increased by over 51%, together with *Bacteroidetes* and unidentified bacteriome. It is in accordance with our previous studies (Chopkova et al., 2023; Petkova & Shilev, 2023) and with the observation of Zhang et al. (2021). Surprisingly, the prokaryote microbiome of TP is quite similar to those in SM concerning the phylum. Changes also were observed in the second mesophilic phase. Thus, its structure was very similar to the MP with increased *Firmicutes*. The vermicompost demonstrated dominancy of *Proteobacteria* over 60%. Huang et al. (2019) reported similar results that *Proteobacteria, Actinobacteria, Bacteroidetes* and *Firmicutes* were the dominant bacterial communities, while *Bacillus* (*Firmicutes*) dominated at the genus level.

Delving deeper into the prokaryotic diversity in compost and vermicompost, genera changed significantly during each phase (Fig. 3b). For example, the source materials show the dominance of genera *Bacillus*, (46%), followed by *Sporosarcina* (8%) and *Galbitalea* (6%). The beginning of composting and the entry into the mesophilic phase led to a complete succession of bacterial communities. The presence of almost 80% unknown species in the

databases is particularly impressive and shows the lack of information on the essence of composting. Those identified were *Stenotrophomonas* (7%), *Bacillus* (6%), *Arenimonas* (5%) and *Paenibacillus* (2%). Domination of *Paenibacillus* and *Solibacillus* in TP was observed. Species of the genera *Solibacillus* and *Planococcus* occur only in TP of composting, which could mean that they are strict thermophiles. Thus we confirmed the results of Zainudin et al. (2014) who found dominance of *Solibacillus silvestris* in thermosphilic phase of composting strongly associated to the lingocellulose degradation occurring mainly in that phase. These authors observed *Planococcus* to be dominant in maturing stage and not in thermophilic, as we found. Other researcher reported that representatives of this species possess genes of cellulose and hemicellulose decomposition (Morohoshi et al., 2012). In addition, our findings are in contrary to those of Finore et al. (2023), which found *Bacillus* and *Thermus* domination role in the thermophilic phase.

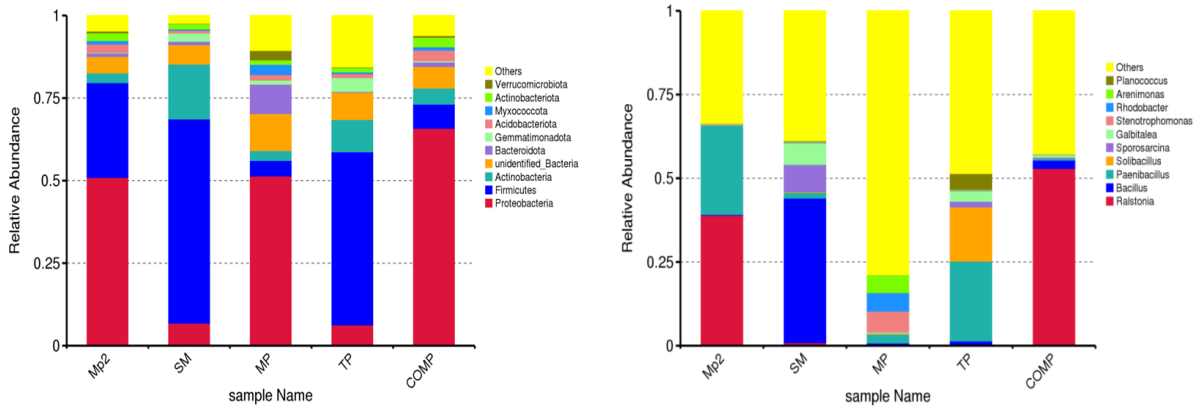


Fig. 3. Relative abundance of phylum (a) and of genus-level taxa in the composting phases (b).

Furthermore, another succession was observed passing to the MP2, where *Paenibacillus* was presented along with *Ralstonia*. In the final vermicompost, *Paenibacillus* disappeared, while *Ralstonia* continued dominating (Fig. 3b). Other researchers reported about the important role of temperature that modified the microbiome structure during composting (Yamamoto et al., 2010). Thus, a strain of *Nitrosomonas europaea* dominated at thermosphilic phase, while the non-cultivable strain of the same genus was found in the mesophilic one. Our results were partially consistent with Tran et al. (2020) that *Bacillus* and *Paenibacillus* were the most dominant bacterial communities in the early phase of composting. In addition, *Stenotrophomonas* was found only in MP2. It is involved in the degradation of DNA, casein, chitin, cellulose and starch, lipids degradation, and nitrate and nitrite reduction (Yang et al., 2006).

In microbial ecology, the study of alpha diversity of amplicon sequencing data is a common method to assess differences between several environments (Willis, 2019). Table 3 shows the richness and diversity of bacterial communities during the composting/vermicomposting process. All pairs were found to be statistically different from each other excepting SM and MP for ACE index. The lowest number of species was found in the TP (2486) followed by the SM (2605). The highest number of 3897 species was observed in the vermicompost, where the abundance was 36.2% higher than TP and 23.6% compared to the MP2. The last showed that vermicomposting contributed 23.6% to the prokaryotic diversity after composting. Diversity (Simpson and Shannon) and richness indices (Ace and Chao1) were calculated to reveal the alpha diversity of composting bacteriome. Shannon and Simpson diversity indices showed

higher values in MP followed by vermicompost in the case of Shannon, which is in line with the findings of Chopkova et al. (2023). Both Chao1 and ACE demonstrated much higher richness in the vermicompost. Our results that bacteriome diversity in wheat straw and cow manure composting increased with the process advancement and reached its peak in the final product are consistent with the findings of other researchers (Fierrer et al., 2010). Wang et al. (2015) found an increasing of α-diversity indices in all composting phases concerning the source materials, highest in the mesophilic phase with 66.4%. The increased taxonomic diversity is closely related to the increased metabolic diversity that may lead to improved organic compounds' degradation and transformation rate during composting.

Table 3. Indices of alfa diversity at each composting phase. Different letters show significant differences among composting phases according Student's t-test ($p<0.05$).

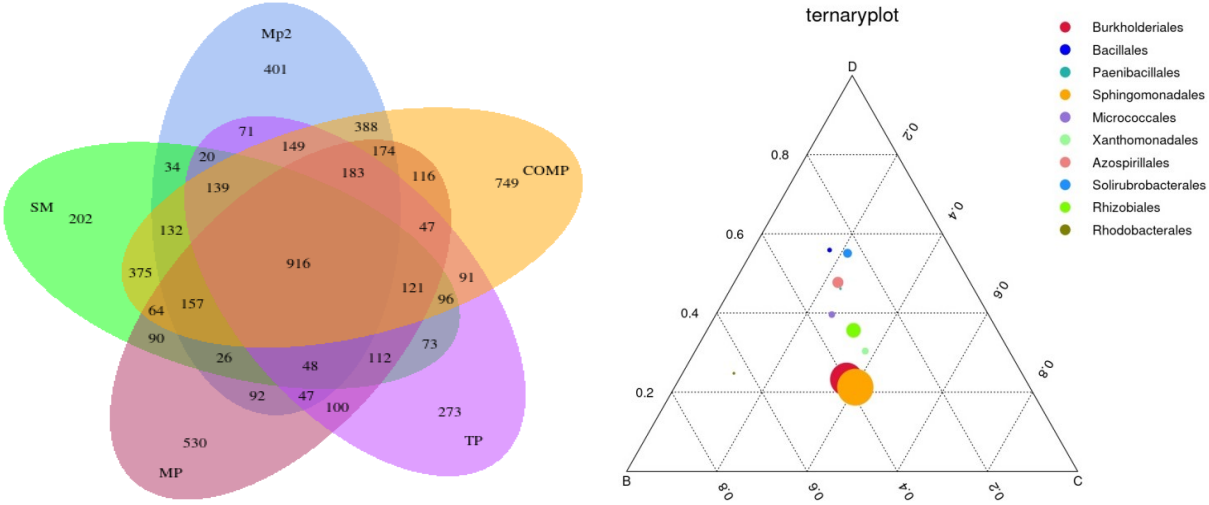
Sample name	observed species	Shannon	Simpson	Chao1	ACE	goods coverage	PD whole tree
SM	2605a	5.152a	0.809a	2995.438a	3058.473a	0.993	231.796
MP	2823b	8.344b	0.987b	3080.903b	3111.601a	0.995	255.221
TP	2486c	5.454c	0.898c	2868.553c	2910.012b	0.994	216.038
MP2	2977d	5.072d	0.782d	3299.887d	3332.816c	0.994	222.079
COMP	3897e	5.752e	0.729e	4449.576e	4583.067d	0.990	285.631

Pearson correlation analysis of physicochemical factors and diversity indices with significance of $p<0.05$ revealed the strong influence of compost temperature on bacteriome diversity in all studied phases. Species abundance was highly dependent of this factor during preparation of composting piles (SM, 0.999, $p<0.05$), while a negative dependency was found between the C:N content and Shannon and Ace indices (-0.992, -0.990). In MP strongest proven positive correlation existed between the species richness (1.000, $p<0.01$), Chao1 (0.998, $p<0.05$) and Ace (1.000, $p<0.05$) indexes, and the temperature. A negative dependence was found between the C:N content from the one hand, and the number of observed species (-0.999, $p<0.05$), as well as the Chao1 (-0.994) and Ace (-1.000, $p<0.05$) indices from the other. At the TP, temperature was the only one of the studied parameters that was in relationships to the bacteriome – positive for the observed species (0.998, $p<0.05$) and Simpson (0.997, $p<0.05$) index and negative in the rest three cases (Shannon, Chao1 and Ace). The species abundance and diversity indices were strongly influenced by phosphorus content in the MP2 (0.999, $p<0.05$; 1.000, $p<0.05$; 0.999, $p<0.05$; 1.000, $p<0.01$; 1.000, $p<0.01$), but negatively correlated to the temperature and pH ($p<0.01$). The Pearson correlation in final vermicompost (Comp) revealed relative good relationships between the species abundance, including diversity indices and the temperature except for the Chao1 index where the dependence was negative.

Venn diagrams are widely used tools for graphically depicting the unions, intersections, and differences between multiple data sets (Venn, 1880; Edwards, 2015). The diagram in Figure 4a compares the bacterial diversity in the straw and cow manure composting phases and the vermicompost. A total of 5783 OTUs were differentially investigated in the five phases. A total of 916 OTUs were shared by all five compost phases SM, MP, TP, MP2 and COMP. A higher unique number of species, characteristic for the corresponding sample, were found in the final product, the vermicompost (749), followed by the MP (530), MP2 (401), TP (273) and SM (202). When comparing source material (SM) versus mature vermicompost, the most significant overlap was observed at 375 species. Ninety species passed from SM phase to MP. In comparing the mesophilic phases (MP and MP2), 92 were common species, and the sum of shared species with the remaining samples was 940, 16.25%. Seventy-one species went from TP to MP2. A

higher similarity between the studied samples was found for MP2 and vermicompost (COMP) – 388 unique species. The lowest common OTUs were found in the SM compared to the second mesophilic phase. This revealed that the number and species showing changes in growth and development across all five developmental phases were distinctive.

The triple plot diagram of the most abundant bacteria (>70% relative abundance in the mesophilic and thermophilic phases of compost) and their distribution between MP, MP2 and TP is shown in Figure 4b. Each circle represents an OTU, and its size represents relative abundance. From Venn diagram is clear that the active composting phases shared 47 species indicating a strong influence of temperature on straw and cow manure bacteriome. Thus, the orders *Burkholderiales* and especially *Sphingomonadales* were the most abundant in the studied phases. Moreover, they were very closely situated to each other and the mesophilic phases. At the same time, they were at an equal distance from MP and MP2, but quite distant from TP. Other bacteriome orders presented in much lower abundance were *Rhizobiales, Azospiralles* and *Solirubrobacterales*. They tended to the thermophilic phase as much closer was order *Solirubrobacterales*. Other orders found in this study were *Bacillales, Xhantomonadales, Micrococcales, Paenibacillales*, and *Rhodobacterales*.



(a) (b)

Fig. 4. Venn diagram depicting associations, intersections and differences between microbial species at the phases of the composting process (a); Tripartite distribution diagram of OTUs between mesophilic (B is MP and C is MP2) and thermophilic phases (TP) of composting (b).

The β-diversity was performed to determine differences and similarities between all composting phases by cluster analysis (Fig. 5a). The quantitative data results of analyzed samples showed the lowest difference in β-diversity values between SM and TP (0.280), and between COMP and MP2 (0.291), respectively. The highest dissimilarity was found when analyzing SM and MP2 (0.885), followed by MP and TP (0.861). High dissimilarity between the vermicompost bacteriome and SM and TP was also observed (0.809). In this sense, the difference between TP and SM was relatively small (0.280). Bacterial communities at the phylum level and β-diversity showed that *Proteobacteria*, *Firmicutes, Bacteroidetes* and *Actinobacteria* were most strongly affected by temperature and respectively by the composting phases (Fig. 3a). Thus, the changes occurred in the bacteriome were due to the adaptation to the changes occurred in the abiotic conditions, as temperature, nutrients and other physicochemical properties, which is in line with the conclusions reported by several authors (Hou et al., 2019; Kitamura et al., 2021; Li et al., 2022).

To investigate the similarity between different samples, cluster analysis based on the unweighted group pair method with arithmetic mean (UPGMA) was used to construct a cluster tree in Figure 5b. The SM cluster and the TP, where *Firmicutes* bacteria predominate and *Proteobacteria* and *Actinobacteria* had a significant role in the degradation processes. The second cluster obtained by the unweighted pair method included early and late mesophilic phase bacteria and mature vermicompost. Similar to the results of Figure 3a, the species belonging to *Proteo-bacteria* had the strongest influence, followed by *Firmicutes*.

Initial PCoA showed a significant weight (PC1-68.16% and PC2-23.46%) of the total variation of bacterial taxa at the phylum level obtained from the study of all five phases of composting-vermicomposting technology (Fig. 6a). The results show that the sequences in SM and TP form one cluster, MP2 and COMP cluster together. The initial meso-philic phase bacteriome was quite distant from the rest of the samples (Fig. 6b).

The second PCoA showed variation within PC1-35.93% and PC2-29.25% of the total variation in biodiversity at the type level among all five compost samples where TP and MP were grouped.

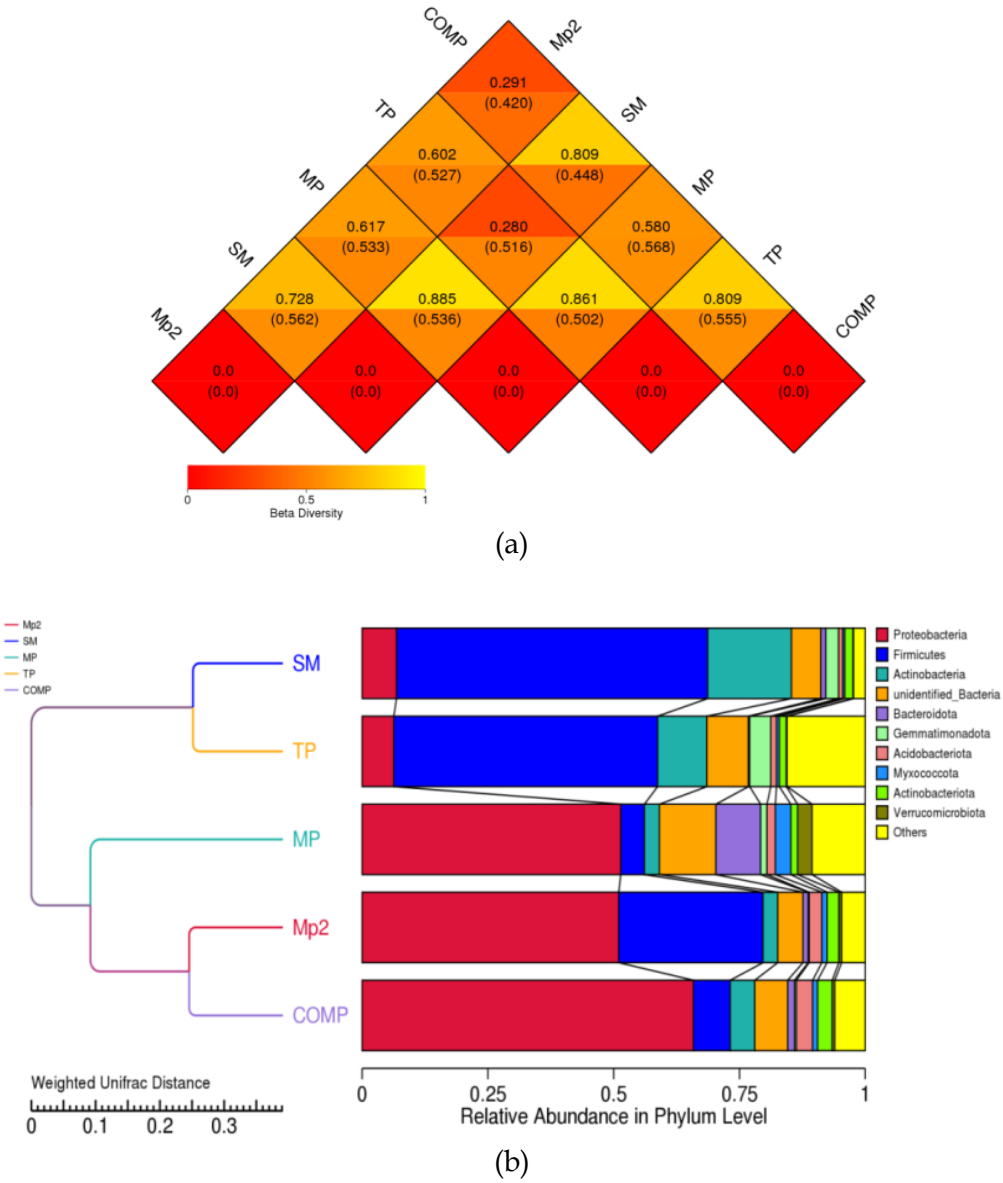
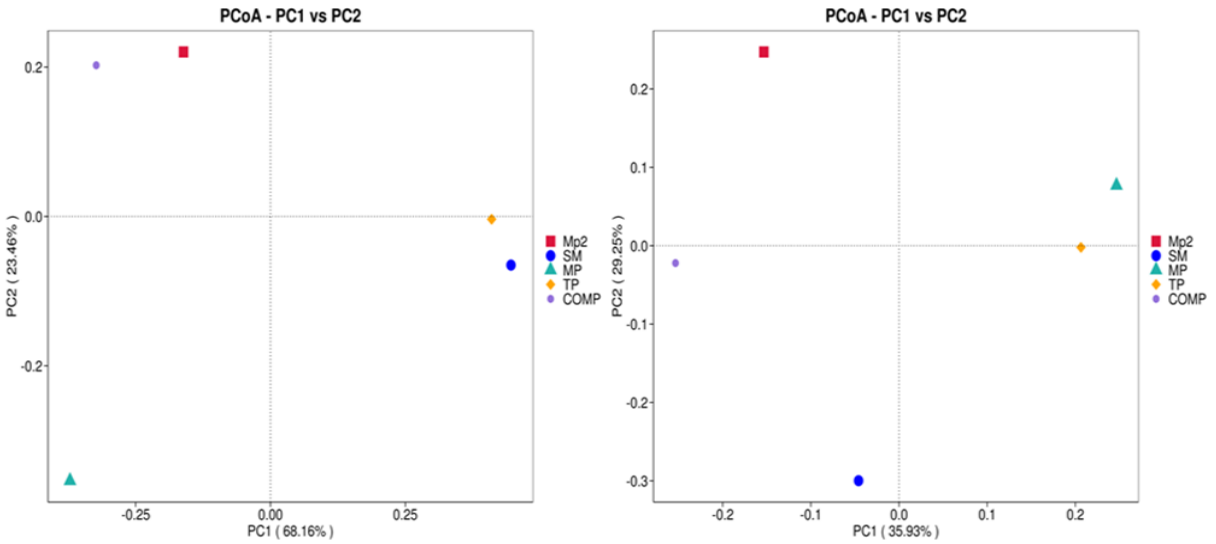


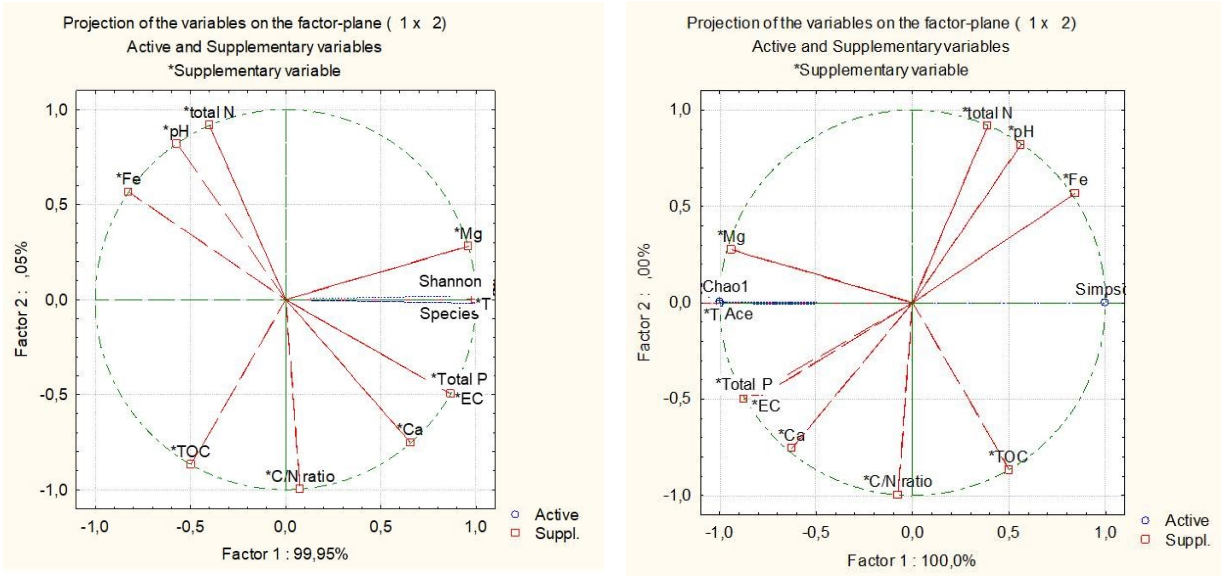
Fig. 5. Bacterial beta-diversity map of interrelations among composting phases (a) and UPGMA dendrogram and clustering (b).



a) b)

Fig. 6. PCoA based on weighted Unifrac distance (a) and based on Euclidean distances for applying variance decomposition to reduce the dimensions of multivariate data (b).

The temperature was the basic factor influencing the prokaryotic diversity in composting piles (Fig. 7a). Principal component analysis (PCA) clearly demonstrated that the Factor 1 was the most important with weight of 99.95%. Thus the number of observed species and the Shannon diversity index were strongly depended from it. The Factor 2 did not show any influence on the main variables. In addition, the temperature changed negatively influenced the Chao1 and Ace indices, but positively the Simpson index (Fig. 7b). Total phosphorus and EC were grouped together showing positive correlation with the temperature. In addition, the total nitrogen and pH were situated very closely to each other, but negatively influenced by the pile temperature.



(a) (b)

Fig. 7. PCA of the influence of the factors on the number of species and the Shannon index (a) and on the Simpson, Chao1 and Ace indices (b).

Conclusions

Taxonomic features of the composting bacteriome depended on both the abiotic conditions and the nutrients in microhabitats. Temperature had major role in the composting bacteriome changes influencing positively species abundance, Shannon and Simpson indices, and negatively Ace and Chao1 ones. *Firmicutes* and *Actinobacteria* dominated the prokaryotic microbiome in the source materials and in the thermophilic phase, while *Proteobacteria* was the dominated phylum in both mesophilic phases and in the final vermicompost. Thus PCoA and UPGMA dendrogram grouped together SM and TP, by one site, and MP, MP2 and COMP, by another, showing very good similarity. Our future research will aim to uncover the bacterial communities' metabolic pathways and physiological profiles during the composting and vermicomposting of agricultural wastes.

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