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CHARACTERIZING THE BACTERIAL COMMUNITY DURING AEROBIC STABILIZATION OF LIVESTOCK MANURE. WHY SHOULD FEEDSTOCK NOT BE AGED BEFORE COMPOSTING?

The compositions of bacterial communities and populations during aerobic stabilization of livestock manure have been investigated, focusing on how the aging of feedstock affected the bacterial diversity of the composting mass. The livestock manure was divided into two groups – aged and fresh, and then used to prepare the feedstock with additives. Composting experiments were carried out for 15 days using a pilot-scale batch reactor with vacuum-induced aeration. Two different aeration rates were applied to the batch reactor, and their effectiveness was evaluated. Changes in total heterotrophic bacteria count and moisture content were monitored. The associated changes in bacterial community compositions were characterized using 16S ribosomal ribonucleic acid (rRNA) gene sequencing. The *Firmicutes* in the fresh manure decreased from 48 to 13% for the first ten days, and the dominant phylum shifted to the *Proteobacteria* (29%), *Bacteroidetes* (23%), *Actinobacteria* (20%), and others (15%). Under the given conditions, the use of relatively fresh manure was essential to preserve the diverse bacterial populations in the feedstock and enhance the bacterial diversity during aerobic stabilization. More research should be performed to investigate the degradability of emerging contaminants (*e.g.*, antibiotics) in livestock manure using an engineered composting system providing well-controlled environmental conditions.

1. INTRODUCTION

We recently noticed that a remarkable amount of veterinary antibiotics could be released into public water sources (natural surface water) *via* non-point discharge from the

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compost that is extensively used for soil conditioning in suburban areas. The persistent existence of antibiotics is of concern because of the possible ecological impact (e.g., endocrine disruption and antibiotic-resistant species) on biota within the environment [1, 2]. We have periodically investigated the end products from one of the local treatment plants, which convert livestock manure into a useful soil conditioner. From the periodical investigations, seven antibiotic compounds remaining in the ready-to-use products have been identified. Sulfonamide antibiotics (e.g., sulfamethoxazole and sulfadiazine) were dominant among the residues, and their concentrations ranged from 5 to 144 μg/kg (Table 1). They were derived from the livestock manure used as raw material for the composting. Regardless of whether or not specific antibiotic compounds could be biodegradable, this result recalled us to the robustness and consistency of the manure composting facilities.

Table 1

Veterinary antibiotics remaining in the ready-to-use compost produced from the Bosung Resource Recycling Center (Jeollanam-do, Korea)

Classification	Chemical compound [μg/kg]	S1	S2	S3	S4	S5
Cephalosporins	ceftiofur	–	0.31	–	–	–
Sulfonamides	sulfathiazole	4.9	–	–	–	–
	sulfamethoxazole	120	–	–	–	–
	sulfamethazine	–	–	4.8	1.4	–
	sulfadiazine	144	–	–	–	–
Diaminopyrimidines	trimethoprim	–	–	–	580	33
Tetracyclines	oxytetracycline	–	–	–	1.3	1.3

Compost results from aerobic stabilization of the organic compounds in solid wastes [3, 4]. In composting facilities, raw manure is stacked onsite and used based on a first-in, first-out principle, which may lead to undesirable aging of the material depending on the capacity and proposed retention time. In South Korea, open windrow (or static pile) composting has been mostly replaced with enclosed systems to minimize the effects of off-gasses and flying dust on neighboring communities. Therefore, many composting treatment plants may all be facing material aging or storage issues [5], depending on seasonal variations in the balance between supply and demand. Such uncontrolled aging of manure may occur in livestock farms as well. It is worthwhile to note that the aging of manure as a habitat for microorganisms readily changes its biophysiological characteristics and thus affects the organic stabilization and contaminant degradation during composting.

Composting aerobically decomposes organic matter and is affected by several factors, including feedstock composition, moisture, oxygen, and temperature. This study focuses on the bacterial diversity of the feedstock, which could be enhanced during the course of composting, depending on the freshness of the livestock manure collected. The diversity and richness of the microbial community have been emphasized, especially to achieve a robust

removal of the contaminants that are of growing concern. Wang et al. [6] reported that enhanced microbial diversity promoted the removal of antibiotics and organics during sludge treatment with phytoremediation. Zhou and Yao [7] suggested that the diversity and abundance of bacterial communities affected by composting conditions were the main drivers of reducing antibiotic resistance genes. During the composting process, the diversity of microbial populations also allows the decomposition of a wide range of material from simple, easily degradable material to more complex, decay-resistant matter [8]. For manure composting done using diverse microorganisms, more importantly, an elevated temperature can accelerate the degradation of antibiotic compounds because of greater biological activity or thermal-driven abiotic degradation [9].

Numerous studies have been done on engineered composting systems, focusing mainly on the stabilization efficiency of organic matter and dealing also with how bacterial communities change in the middle of composting [10–12]. Given the critical role of bacteria as a decomposer, however, relatively little information is available on how feedstock freshness affects the diversity and populations of microorganisms found in the compost after achieving thermophilic temperatures. In the context of a better degradation of emerging contaminants, it will be beneficial to ensure diverse microbial compositions in the compost during the entire course of aerobic stabilization [10]. Thermophilic temperatures typically indicate that intense microbial activity is taking place [8], which is arguably the most important phase of composting in terms of process control. The temperature continues to increase steadily as the microbial population increases, but reaching the thermophilic stage of composting does not mean microorganisms in the compost always diversify into some populations. Thus, the main hypothesis to be tested was that aging of manure for any reason (until it is fed to the composting reactor) could harm the bacterial community and disable restoration of the bacterial diversity during the composting process. In this study, we characterized the bacterial community and monitored its compositional distribution during aerobic stabilization of the feedstock prepared with aged and fresh manure. Understanding how aging affects microbial composition would be beneficial for saving processing time and energy for composting effectively, as well as for removing the contaminants of emerging concern.

2. MATERIALS AND METHODS

Preparation and characterization of the feedstock. Pig, chicken, and cow manure samples were collected from the Bosung Resource Recycling Center (Jeollanam-do, Korea), which converts livestock manure into a soil conditioner using enclosed composting systems. We divided manure samples into two groups, i.e., aged samples stored in the yard for more than a week because of lack of land space and fresh ones collected immediately after transport from a local livestock farm. The pig, chicken, and cow manure were mixed at a weight ratio of 4:4:2. The mixture was then supplemented with

sawdust at different rates and subsequently inoculated with a consortium of microorganisms before composting. Bacterial phyla of the selected inoculum comprised *Actinobacteria* (43%), *Proteobacteria* (29%), *Bacteroidetes* (19%), *Firmicutes* (2%), and others (7%). Characteristics of the feedstock prepared for composting and the experimental conditions are summarized in Table 2. Moisture content was measured based on the weight loss of the samples at 105 °C for 24 h [10]. The dried samples were homogenized and then burned in a furnace (550 °C for 2 h) to measure the volatile solids (VS). Total carbon (%) and total nitrogen (%) were analyzed to obtain the C/N ratio using a CHNSO elemental analyzer (Elementar/Vario Micro cube, Germany) in dry matter. We also analyzed *Salmonella* and *E. coli* O157:H7 using real-time PCR.

Table 2

The characteristics of the feedstock prepared for the composting experiments and the experimental conditions for each of the composting runs

Item	C1	C2	C3	C4	C5	C6	C7
Manure condition[a]	aged					fresh	
Inoculation	no	yes					
Supplement rate[b], wt. %	0	0	5	27	43	0	3
Moisture content, %	65	66	45	37	35	54	59
C/N ratio	21	20	34	43	41	29	23
Aeration rate, $dm^3/(h \cdot kg\ VS)$	36	36	270				

[a]The term aged designates manure that was usually stored in the yard for 1–2 weeks before initiating composting processes, whereas relatively fresh manure (termed fresh) was collected immediately after transport from a local livestock farm; thereby, the term fresh does not mean that manure just excreted has been used in the experiments.

[b]Sawdust was used as a supplement to reduce moisture content and increase the porosity of the feedstock for effective aeration.

Composting conditions. In South Korea, open windrow facilities are increasingly retrofitted with well-engineered reactor systems not only to save energy and costs but also to minimize leachate and odor emissions. We designed a containerized batch reactor according to such commercial requirements and operated it for the composting experiments using feedstocks with different characteristics (Fig. 1). In this study, we carried out seven runs of composting for 15 days using the containerized batch reactor ($L \times W \times H$ = 3.0×2.2×2.5 m) with an effective working weight of 600 kg. Vacuum-induced aeration was applied to the reactor and evaluated at two different rates (36 and 270 $dm^3/(h \cdot kg\ VS)$ of the feedstock) to control airflow and material temperature during composting. The aeration was done four times a day for 10 min each while agitating the composting material. During the course of the composting experiments, the temperature of the material was recorded every 6 h to identify the thermophilic phase of composting.

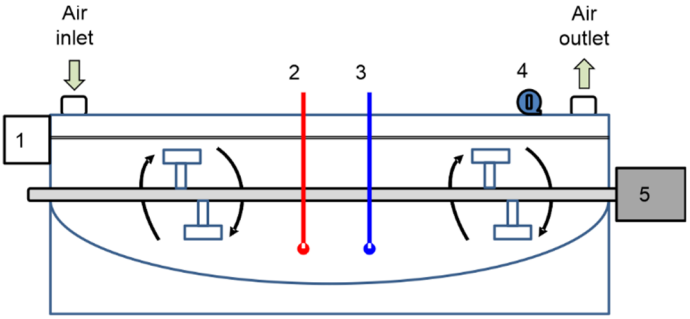

Fig. 1. Schematic of a containerized batch reactor for manure composting:
1 – control box, 2 – thermometer, 3 hygrometer, 4 – vacuum blower, 5 – motor drive shaft

Microbial assay. Compost samples were collected on days 0, 10, and 15 and subjected to the microbial assay. The total heterotrophic bacterial count was measured using the standard plate counting method for which plates were incubated in an inverted position at 35 °C for 72 h [13]. The microbial community was characterized using 16S ribosomal ribonucleic acid (rRNA) gene sequencing and PCR amplification was performed using the 16S rRNA gene with extracted DNA. We carried out amplifications at an initial denaturation at 95 °C for 3 min, followed by 25 cycles of denaturation at 95 °C for 30 s, primer annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, with a final elongation at 72 °C for 5 min. Then, secondary amplification for attaching the Illumina NexTera barcode was performed. The PCR product was confirmed by using 2% agarose gel electrophoresis and visualized under a Gel Doc system (BioRad, Hercules, CA, USA). The amplified products were purified with the QIA quick PCR purification kit (Qiagen, Valencia, CA, USA). We pooled equal concentrations of purified products together and removed short fragments (non-target products) with an Ampure beads kit (Agencourt Bioscience, MA, USA). The quality and product size distribution were assessed on a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) using a DNA 7500 chip. Mixed amplicons were pooled and sequencing was carried out using a MiSeq system (Illumina, USA) according to the manufacturer's instructions at Chunlab, Inc. (Seoul, Korea). The CLCommunityTM software (Ver. 3.46) was used for data analysis and visualization.

Antibiotic detection. All chemicals used to measure the selected antibiotics were supplied by Sigma-Aldrich (St. Louis, MO, USA). The antibiotics were extracted by solid-phase extraction (OASIS HLB cartridge) and analyzed using an Agilent 1200 high-performance liquid chromatography. The chromatographic separation was carried out on a Zorbax Eclipse Plus C18 column with mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in methanol). Mass spectrometry detection was performed on an Agilent 6460 triple-quadrupole mass spectrometer equipped with a dual jet-stream electrospray ionization source. Quantitative and qualitative analysis was carried out for the selected antibiotics by multiple reaction monitoring.

3. RESULTS AND DISCUSSION

3.1. EVALUATION OF FACTORS AFFECTING THE TEMPERATURE OF COMPOST

Composting is an exothermic process and uses oxygen as the electron acceptor to maximize the energy release during the aerobic decomposition of organic matter [3]. A sufficient air supply is thus essential to boost organic decomposition and increase the temperature of the composting material. Figure 2 shows changes in the temperature of the composting material during the aerobic stabilization of the livestock manure using two different aeration rates (36 and 270 $dm^3/(h \cdot kg\ VS)$). For composting of aged manure with indigenous microorganisms (C1) at an aeration rate of 36 $dm^3/(h \cdot kg\ VS)$, the material temperature was higher than the ambient temperature (15±2 °C), but we observed no indication of high-rate degradation. Interestingly, composting of the inoculated feedstock (C2) achieved a peak temperature of 54 °C in a day, which was probably attributed to metabolic heat production caused by enhanced microbial activity compared to C1. However, the air supply (36 $dm^3/(h \cdot kg\ VS)$) applied to C2 was not sufficient to boost microbial decomposition of the organic matter in the feedstock, and thus the material temperature quickly decreased to <30 °C within the first three days of composting. Successful composting requires an environment that has sufficient oxygen supply, moisture, carbon, and nutrients to support maximum microbial growth [14].

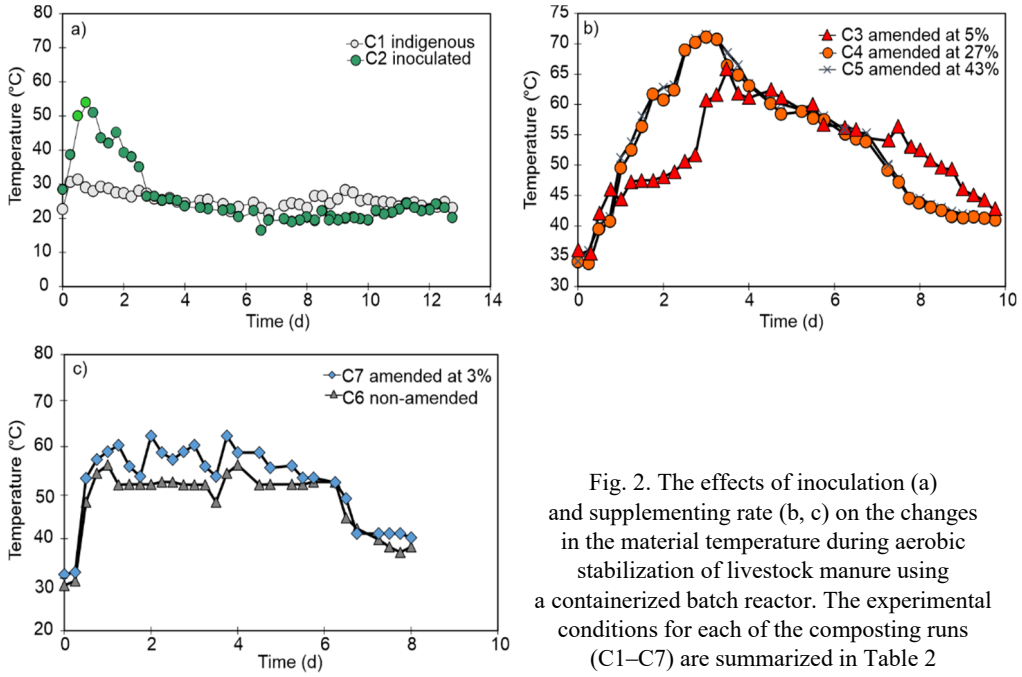

Fig. 2. The effects of inoculation (a) and supplementing rate (b, c) on the changes in the material temperature during aerobic stabilization of livestock manure using a containerized batch reactor. The experimental conditions for each of the composting runs (C1–C7) are summarized in Table 2

During composting at an aeration rate of 270 $dm^3/(h \cdot kg\ VS)$ (C3–C7), the composts heated to temperatures in the pasteurization range of 50 to 70°C (Figs. 2b–d), which could destroy the enteric pathogenic organisms. Such vigorous heating implicates intense biological activity in the composts, and the microorganisms responsible for composting also degrade a broad range of organic compounds [8]. Fresh manure was used for C6 and C7, whereas the other composting runs were operated with "aged" manure. Both C6 and C7 achieved compost temperatures above 50°C within a day of composting and maintained them for six days. Similarly, C3–C5 maintained high temperatures above 50 °C for six days; however, they needed a longer time to reach more than 50 °C than when fresh manure was used (C6 and C7). For composting with "aged" manure, the time to reach more than 50 °C was shortened by increasing the supplement from 5% (C3) to 27% (C4), but further increasing the supplementing to 43% (C5) resulted in no difference in the temperature profile of composting compared to C4. The addition of dry supplements, such as sawdust and rice hulls, is a common way to increase the porosity of the mixture for effective aeration and lower the initial moisture content. However, the use of a supplement increases the compost mass and tends to slow down the microbial degradation because of the higher C/N ratio [15]. The compost temperature decreased to around 40 °C on day 8 for fresh manure or day 10 for aged manure (C3–C5). Because environmental factors (e.g., aeration, moisture, and temperature) do not limit microbial activity, the decrease in material temperature was attributed to a depletion of biodegradable fractions in the feedstock, indicating stabilization of the compost.

3.2. CHARACTERIZATION OF THE BACTERIAL COMMUNITY DURING COMPOSTING

Based on the temperature profiles monitored in this study, one could judge the composting of C3–C7 to be up to standard, but the robustness of composting may depend on the feedstock conditions, as hypothesized in the introduction. Therefore, this study further diagnosed C3, C4, and C7 and evaluated them in comparison with C2 as a negative control. During composting of C3, C4, and C7, the material temperature increased to over 50 °C and was maintained for six days. This thermophilic composting significantly increased the bacterial populations (Fig. 3), along also with the change in the compositional distribution of the bacterial community (Fig. 4). Those changes coincided with an evaporative release of water from the compost. The increased temperature during composting reduces the moisture content *via* evaporation, resulting in increased oxygen transport through the pores between particles in the composting mass [16]. Despite the small amounts of water that are generated as a metabolic end product, how the moisture content decreases during composting is especially dependent on the temperature of the compost and the aeration rate. The bacterial populations in the feedstock of C2 were significantly decreased during composting, probably because of limited oxygen transport, as was consistent with a negligible change in the bacterial community compositions. An anaerobic condition maintained by limiting oxygen transport would inhibit

microbial proliferation. Considering the applied aeration rate and temperature during composting, this is not surprising, as discussed previously. The composting temperature has been reported to be one of the most influential factors for microbial activity elsewhere [17–19].

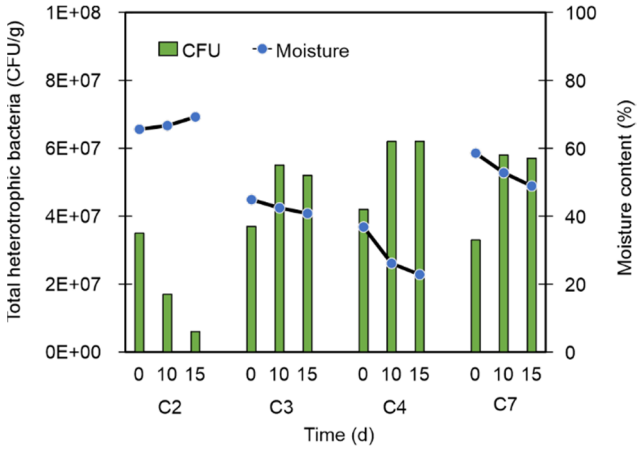

Fig. 3. The changes in total heterotrophic bacterial count and moisture content during aerobic stabilization of livestock manure under different composting conditions

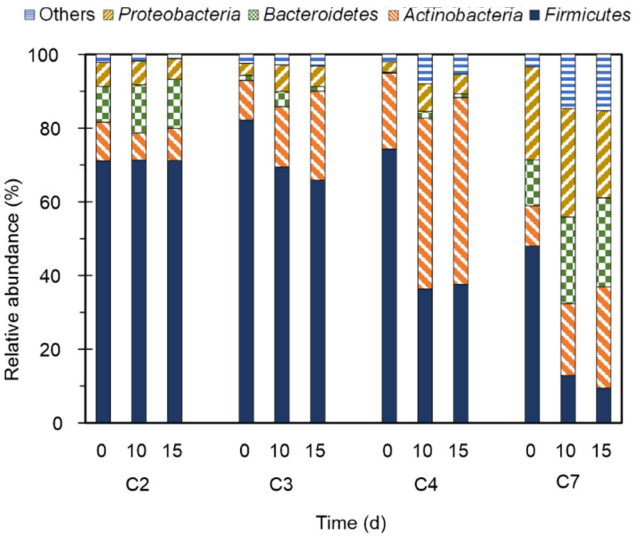

Fig. 4. The compositional distribution of the bacterial community identified in the composting material during aerobic stabilization of livestock manure under different composting conditions

Figure 4 shows that the *Firmicutes* were the dominant bacteria in the feedstock regardless of its freshness and declined to various extents during the composting. The use

of relatively fresh manure (C7) preserved diverse bacterial populations, and the bacterial diversity in the feedstock dramatically improved during the aerobic stabilization, which also changed the distribution among the identified bacterial phyla. The *Firmicutes* in the fresh manure (C7) decreased from 48 to 13% for the first ten days, and the dominant phylum shifted to the *Proteobacteria* (29%), *Bacteroidetes* (23%), *Actinobacteria* (20%), and others (15%). *Rhodothermaeota* and *Deinococcus-Thermus* were less than 1% for the initial feedstock but increased up to 13% after 10–15 days of composting. The two phyla were also the extreme minority (<1%) in the feedstock of aged manure (C3 and C4), and neither of those increased noticeably during composting for the given time. For composting aged manure (C3 and C4), the decrease of the *Firmicutes* was greater when more supplements were used under the given conditions. C3 showed that the *Firmicutes* decreased by 13–16% over 10–15 days and still occupied the dominant position in the compost, whereas C4 showed a significant change in the bacterial community. For the composting of C4 under the given conditions, the *Firmicutes* decreased from 74 to 36–38%, while the *Actinobacteria* increased from 21 to 46–51%. By increasing the amount of supplement from 5% (C3) to 27% (C4), the dominant *Firmicutes* were displaced by the *Actinobacteria* in the first 10 days, but the sum of the other phyla was still lower than 18% during the composting process. This result indicates that once the feedstock has aged for 1–2 weeks before initiating composting processes, it might be difficult to restore a diverse compositional distribution of the bacterial community even with the input of additional energy, time, and additives. A variety of microbial populations develop in response to the freshness of the collected livestock manure. This microbial diversity enables the composting process to continue despite the constantly changing environmental and nutritional conditions within compost [8]. Although piling the composting with less mixing for a long period is beneficial in some cases, such as for nitrogen fixation, the restoration of bacterial diversity in the thermophilic phase is needed to achieve effective stabilization of livestock manure when using controlled composting systems.

4. CONCLUSIONS

It is of great importance to secure the diversity and abundance of the microbial communities in the biological treatment process. In this study, we investigated how aging affected the bacterial diversity and populations of raw manure during the manure composting. Our results indicated that the aging of the raw manure as a microbial habitat deteriorated the bacterial diversity of the feedstock and consequently disabled the restoration of diverse bacterial communities despite the replaced dominant phylum during the thermophilic degradation of organic matter. This can also worsen organic stabilization and contaminant degradation during the composting process. The robustness of composting has been typically judged based on the temperature profile of composting, which could be done better by also using a bioassay technique for a better interpretation

of the treatability. More research should be performed to characterize the fate of emerging contaminants (e.g., antibiotics) remaining in the livestock manure under well-controlled composting conditions, and more work should also be done on our proprietary composting system to increase its economic feasibility by saving energy input.

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