Contributions of Thermotolerant Bacteria to Organic Matter Degradation under a Hyperthermophilic Pretreatment Process during Chicken Manure Composting

Yun Cao,[a,b,c] Lin Wang,[a,d] Yuting Qian,[a] Yueding Xu,[a,b,c] Huashan Wu,[a,b,c] Jing Zhang,[a,b,c] Hongying Huang,[a,b,c,*] and Zhizhou Chang [a,b,c]

Composting technology comprising hyperthermophilic pretreatment (at ⩾ 85 °C for 2 to 4 h, HTPRT) and aerobic composting was adopted to accelerate organic matter transformation and enhance nitrogen retention in chicken manure composting. The differences in physio-chemical parameters, successions, and metabolism functions of the bacterial community between HTPRT (85 °C, 4 h) and conventional composting (CK) were compared. The HTPRT composting system reached maturity 18 days in advance of CK. The HTPRT piles showed a lower maximum N loss (27.1% *vs.* 39.0%). The bacterial structure in the HTPRT system differed remarkably from that in CK. *Ureibacillus* (22.7%) and *Ammoniibacillus* (14.1%) were the most predominant species in the thermophilic phase of HTPRT pile, while the curing phase was dominated by *Thermobifida* (12.8%) and *Saccharomonospora* (11.8%). The authors' results suggest that HTPRT improved the physical properties of the feedstock by reducing the bulk density, which favored microbiological activity, and thus improving composting efficiency.

Keywords: Hyperthermophilic pretreatment; Animal manure waste; Nitrogen retention; Thermotolerant bacteria; Microbial community

Contact information: a: Circular Agriculture Research Center, Jiangsu Academy of Agricultural Sciences, Nanjing 210014, P.R. China; b: Key Laboratory of Crop and Livestock Integrated Farming, Ministry of Agriculture, Nanjing 210014, P.R. China; c: Jiangsu Collaborative Innovation Center for Solid Organic Waste Resource Utilization, Nanjing 210014, P.R. China; d: Nanjing Institute of Agricultural Sciences in Jiangsu Hilly Area, Nanjing 210046, P.R. China;
** Corresponding author: youngtsao66@126.com*

INTRODUCTION

With the increase in the intensive livestock production, the quantity of poultry waste has dramatically increased in recent years (*China Statistical Yearbook* 2016; Zhang *et al.* 2016a). Composting is regarded as an effective technology for converting organic wastes, such as poultry manures, into usable organic fertilizers or a soil amendment. However, in some cases, the physio-chemical characteristics of organic wastes may make the wastes unsuitable for conventional composting. These properties may result in a lower temperature during composting and cause insufficient decomposition and poor sanitation of the composts. Moreover, the composting process can involve a considerable generation of malodourous gases such as ammonia (NH_3), hydrogen sulfide, and volatile organic compounds (Jurado *et al.* 2015). It is estimated that ammonia emissions contributed to 71% to 88% of nitrogen loss in composting facilities (Steiner *et al.* 2010).

Composting is a self-heating aerobic process driven by the actions of microbial communities (Gannes *et al.* 2013a). These communities are affected by the types of substrates present and environmental conditions, such as temperature, ventilation conditions, and pH (Tortosa *et al.* 2017). The process of composting is generally comprised of three predictable phases, mesophilic, thermophilic, and curing (Gannes *et al.* 2013a; Zhang and Sun 2014). These temperature phases reflect the activities of successive microbial populations responsible for the degradation of organic matter. As a result, temperature is regarded as one of the crucial variables during transformation of organic matter (OM). To promote the efficient OM degradation, the composting process is expected to continuously remain in the thermophilic phase. Thus, various countermeasures have been used to increase the temperatures during the composting of animal manures: aeration control, bulk agent amendment (Jurado *et al.* 2015; Zhou *et al.* 2015; Zhao *et al.* 2017; Liu *et al.* 2019), and direct microbial inoculation (Zhao *et al.* 2017; 2018). In fact, compost conditions, such as the pH, C/N ratio, and oxygen concentration improvement, have significant influence on the growth of microbial communities (Zhang and Sun 2014; Tortosa *et al.* 2017).

Recently, composting of organic wastes by using a hyperthermophilic pretreatment (HTPRT) process has been developed by Yamada *et al.* (2007) to solve malodorous odor problems related to ammonia emission during the composting process. During the process, feedstocks first were subjected to a HTPRT reactor followed by a conventional composting system. The temperature of the raw materials in the HTPRT reactor reached 100 °C, and the maximum temperature was kept for at least 30 min. The HTPRT has been reported as a novel technology that has prominent features, such as high bioconversion efficiency, accelerated formation of humic acids, low ammonia emission, and accelerated removal of antibiotic resistance genes and mobile genetic elements during composting (Liao *et al.* 2018).

Although there have been some reports concerning composting combined with an HTPRT process, the mechanism by which the HTPRT process stabilizes animal manures in the subsequent aerobic composting system remains to be further researched. Previous studies mainly have focused on the dynamic changes in the composition of the microbial communities responsible for OM conversion during different composting phases. Few studies have concerned the metabolic activity and functional diversity of the bacterial community, which are of great importance to reveal OM transformation in composting.

The objectives of the study were to: 1) compare the difference in the succession and metabolism function of bacterial community between the HTPRT and conventional composting; 2) evaluate the effect of the HTPRT on organic C degradation during poultry manure composting; and 3) identify the relationship between the environmental factors, including temperature, moisture, ash, total organic carbon (TOC), total nitrogen (TN), ammonium (NH_4^+-N), and nitrate (NO_3^--N) contents, and bacterial composition in the HTPRT composting process.

EXPERIMENTAL

Materials and HTPRT Process

The raw materials of chicken manure and rice straw were obtained from Luhe Animal Experimental Base of Jiangsu Academy of Agricultural Sciences (Jiangsu, China). The rice straw was cut into small pieces ranging from 2 cm to 4 cm. The chemical

parameters of the feedstocks before and after HTPRT are shown in Table 1. In order to match what is being done in a composting facility, the HTPRT process and the subsequent thermophilic composting process were conducted in separated systems. Details of the HTPRT process have previously been described in Cao *et al.* (2018). Briefly, in the HTPRT reactor (400 L, Fig. 1A), about 250 kg of the mixed chicken manure and rice straw were stirred using a blender inside the reactor. The mixed materials were heated by dimethyl silicone oil circulating until the preset HTPRT condition (85 °C) was reached and sustained for 4 h. Compulsory ventilation was set at the rate of 100 L/kg TS • h L^{-1} with a blower. After the temperature of the mixed material declined to ambient temperature, it was transferred to a vessel for the subsequent composting (Fig. 1B).

Table 1. Properties of Raw Materials for Composting

Materials	Moisture (%)	Total Organic Carbon (g/kg)	Total Nitrogen (g/kg)	C/N
Chicken manure	71.2	288	25.62	11.24
Rice straw	9.5	405	6.12	66.17

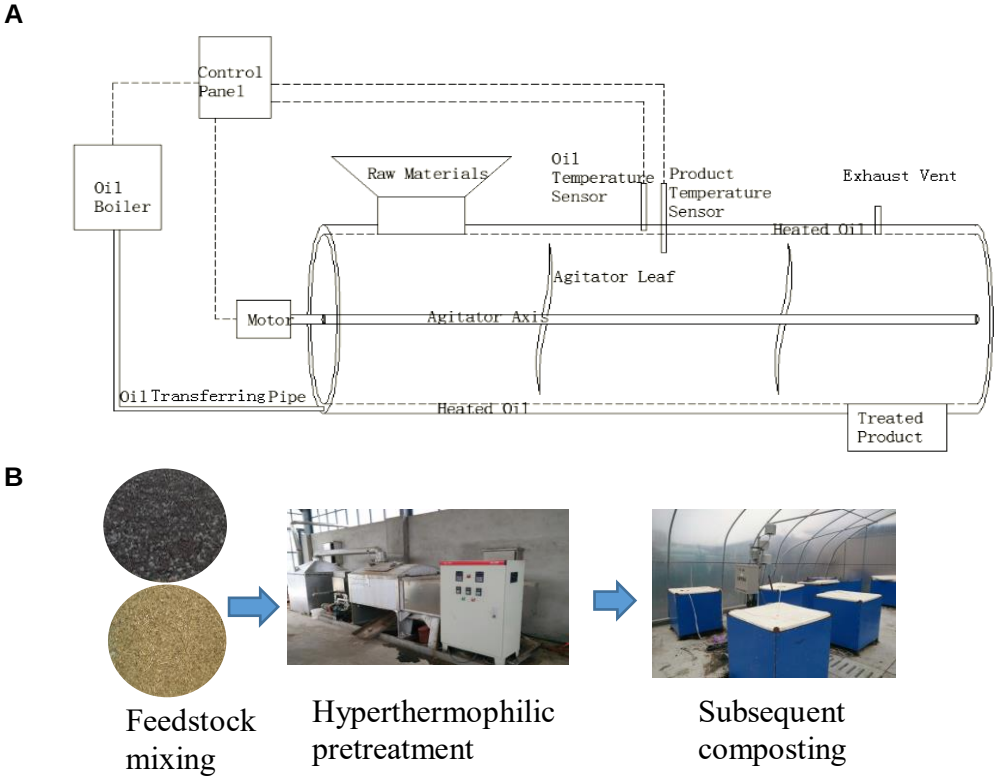


Fig. 1. Schematic diagram of the HTPRT reactor (A) and the HTPRT composting process (B)

Composting setup

The chicken manure and rice straw residues, with or without HTPRT, were adjusted to the same moisture content (62%), and then they were spread on separate plastic sheets and moved into stainless cubic bins (65 cm × 65 cm × 65 cm) with a volume of 300 L for the subsequent aerobic in-vessel composting. The bins were externally insulated with two layers of foam (5 cm in thickness) and aluminum foil thermal insulators to minimize the convective heat loss. A removable foam lid was put on the top of each reactor to facilitate intermittent mixing and sampling of the substrate during composting. A foam board was

fixed on the inner side at the bottom. The uniformly distributed holes (2 cm in diameter) were cut both at the top and the bottom of the bin. The area of the holes accounted for approximately 1/4 of the total area of the foam board. Three thermocouples connected to a data logger were fitted at 15 cm, 30 cm, and 45 cm from the bottom of each bin to record the temperature of the composts at the interval of 60 min. A metal grid with 1 × 1 mm^2 holes was fixed to sustain the composting materials and to allow aeration from the bottom. Fresh air was naturally supplied without forced aeration by turning the composting material manually at 1-week intervals throughout the 62-day-long composting process. While the substrate was being loaded into each bin, original samples were withdrawn (bottom, middle, and top) immediately for subsequent analyses, and the composting time was noted as day zero (Day 0).

Sample collection

A total of 7 solid samples (approximately 250 g) were taken from each bin throughout the composting process at days 0, 7, 14, 21, 28, 42, and 62. Three subsamples taken from the top, middle, and bottom of the reactor were collected and thoroughly mixed. After homogenization, each sample was divided into two parts, with one part kept fresh at 4 °C, and another part air-dried and ground to pass through a 0.25-mm sieve. The air-dried and ground samples were used to analyze the total organic carbon (TOC), total nitrogen (TN), moisture, and ash contents. The fresh samples were used to analyze the pH, ammonium nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N), germination index, and DNA extraction.

Methods

Physio-chemical analysis

The bulk density of the compost was detected using a 100 cm^3 container. The container was filled with samples, and then the samples were slightly compacted to ensure absence of large void spaces. The mass of the compost and container was weighed, and the bulk density was calculated by dividing the weight of the material by the volume of the container (Jain *et al.* 2019). The moisture content was assessed *via* oven-drying at 105 °C for 24 h, and the oven-dried sample was combusted at 550 °C for 6 h in a muffle furnace (ash). The TOC was measured by oxidation with potassium dichromate and TN using the Kjeldahl method on an Automatic Kjeldahl System (Hanon K1100; Hanon Instrument, Jinan, China). The pH was measured in aqueous suspensions of the fresh compost samples (1:5, w/v, compost:water ratio) using a pH electrode (Mettler Toledo, Columbus, OH, USA). The concentrations of NH_4^+-N and NO_3^--N were measured by an Auto Analyzer (AA3; Bran and Luebbe, Werkstraße, Germany). Phytotoxicity to the Chinese cabbage by a Germination Index (GI) test was completed according to the protocol proposed by Zucconi *et al.* (1981).

Microbiological analysis – Enumeration of aerobic microorganisms

Specific culture media and incubation conditions were employed for the enumeration of major microbial groups in the CK and HTPRT piles throughout composting. Fresh samples (10 g) were suspended in 90 mL of sterile water, shaken (170 rpm) at room temperature for 30 min, and standard ten-fold serial dilutions in sterile water were performed. Mesophilic and thermophilic bacteria, actinobacteria, and fungi were cultured in Nutrient Agar (Sigma-Aldrich, St. Louis, MO, USA), Gauze's Synthetic Medium No. 1 (Solarbio, Beijing, China), and Rose Bengal Agar (Sigma-Aldrich, St.

Louis, MO, USA), respectively. The mesophilic microbiota and thermophilic microbiota were incubated at 30 ℃ or 55 ℃ for 2 to 3 days (bacteria), 3 to 4 days (actinobacteria), and 4 to 7 days (fungi) (Liu *et al.* 2017).

DNA extraction

Before DNA extraction, the sample was thoroughly mixed and freeze-dried on a freeze dryer (Alpha 1-2 Ldplus; Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany). Then, the samples were ground with liquid nitrogen to avoid inhomogeneity. The microbial DNA of the samples was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA). Pellets (300 mg) from each sample were collected in triplicate according to the manufacturer's instructions. The DNA quality was assayed using the Nanodrop-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

PCR amplification and sequencing

The V3-V4 region (254 bp) of the 16S rRNA gene was amplified *via* PCR using the following primers:

- Forward: Nobar_341F (CCTACGGGNGGCWGCAG)
- Reverse: Nobar_805R (GACTACHVGGGTATCTAATCC)

The barcode was a 6-base sequence that was inserted between the adapter and the reverse primer so that different samples could be distinguished in just one sequencing run. The PCR reactions were conducted in a 50 μL mixture containing 25 μL premix (Ex Taq, Version 2.0, TaKaRa, Shiga, Japan), 50 ng DNA templates, and 1 μL (10 mM) forward and reverse primers. The mixtures were denatured at 95 ℃ for 5 min, and they were then amplified in 35 cycles of 95 ℃ for 30 s, 55 ℃ for 15 s, and 72 ℃ for 30 s, with a final extension at 72 ℃ for 10 min. The band was extracted and purified using the QIA quick gel extraction kit (Qiagen, Hilden, Germany). After purification and quantification, an equal amount of PCR products from different samples were mixed, purified, and quantified. Combined PCR products were sent to Sangon Biotech Co., Ltd. (Shanghai, China) for Illumina sequencing.

Post sequencing analysis

After demultiplexing the raw FASTQ files, the reads generated from the paired-end sequencing using Mothur v. 1.30.1 software (The University of Michigan, Ann Arbor, MI, USA) were combined. Then, the primers, barcodes, and adaptors were trimmed off. Reads shorter than 200 bp were discarded. The quality filtering was also performed using Mothur, discarding reads with ambiguous sequences. The UCHIME program was used to identify and remove chimeric sequences. The sequences that contained more than one ambiguous base or had homopolymers were filtered using USEARCH (version 5.2.236, Sangon Biotech, Shangha, China). Reads shorter than 200 bp were also discarded.

The remaining sequences ("clean sequences") were clustered into OTUs with 97% similarity cut-off using USEARCH. The QIIME program (version 1.9.0, Sangon Biotech, Shanghai, China) was used to assign taxonomy to OTUs based on the Silva-compatible reference alignment (version 119, Sangon Biotech, Shangha, China) at a set confidence threshold of 80% to generate an OTU table. Canonical correspondence analysis (CCA) was performed to analyze the relationships between species diversity and physio-chemical parameters using the Canoco 4.5 software package (Microcomputer power, Ithaca, NY, USA).

Statistical analyses

A one-way analysis of variance was conducted to test the differences in the physio-chemical properties among different composting times. Losses of OM and TN were calculated from the initial (A_1) and final (A_2) ash contents, and the initial (P_1) and final (P_2) fraction concentrations according to the equation of Benito *et al.* (2003) (Eq. 1):

$$\% \text{ Loss} = 100 - [100 \times (A_1 \times P_2) / (A_2 \times P_1)] \quad (1)$$

All statistical analyses employed the SPSS software (SPSS 19.0, IBM, Armonk, NY, USA). The data were processed using Microsoft Excel (Microsoft Corporation, 2007, Redmond, WA, USA) and all diagrams were plotted with Origin 9.0 (OriginLab Corporation, Northampton, MA, USA).

RESULTS AND DISCUSSION

Effects of HTPRT on Composting Performance

The temperature profiles of the two composting treatments were different (Fig. 2a). The HTPRT rapidly activated the composting process. The temperature of the HTPRT system rapidly increased to approximately 50 °C after 8 days of composting and reached peak temperature at approximately 70 °C on day 10, while in the CK the temperature rose at a much slower rate and reached 50 °C after 11 days of fermentation. This was consistent with previous studies using hyperthermophilic composting technology to process sewage sludge (Liao *et al.* 2018; Yu *et al.* 2018) and waste materials for local communities in Japan (Kanazawa *et al.* 2008). Liao *et al.* (2018) and Yu *et al.* (2018) registered 20 °C to 30 °C higher temperatures during the thermophilic stage than conventional composting because the HTPRT shaped a distinct microbial community that released the metabolic heat during bacterial fermentation.

The HTPRT also affected the physical properties of the composting mixture (Table 2). During the HTPRT, the color of the feedstocks changed from dark yellow to brown. The moisture content for the HTPRT product decreased from 62% to 57%, due to the moisture loss during the pretreatment process. The large size clumps were broken into smaller particles due to the stirring of the feedstocks by the agitator, resulting in a decreased bulk density of the initial feedstocks, which suggested that HTPRT reduced the probability of the formation of anaerobic sites and facilitated O_2 diffusion through the composting mixture (Sánchez-García *et al.* 2015).

Table 2. The Physical Characteristics of Composts from CK and HTPRT Systems

	Moisture Content (%)		Bulk Density (g/cm^3)		pH		Ash (%)	
	CK	HTPRT	CK	HTPRT	CK	HTPRT	CK	HTPRT
Before HTPRT	57.6a	57.6b	0.27b	0.27b	8.13b	8.13b	41.19b	41.19b
After HTPRT	57.6a	62.3a	0.27b	0.24c	8.13b	6.86c	41.19b	38.85c
After In-vessel Composting	22.2b	29.0c	0.36a	0.31a	8.67a	8.65a	56.57a	63.28a

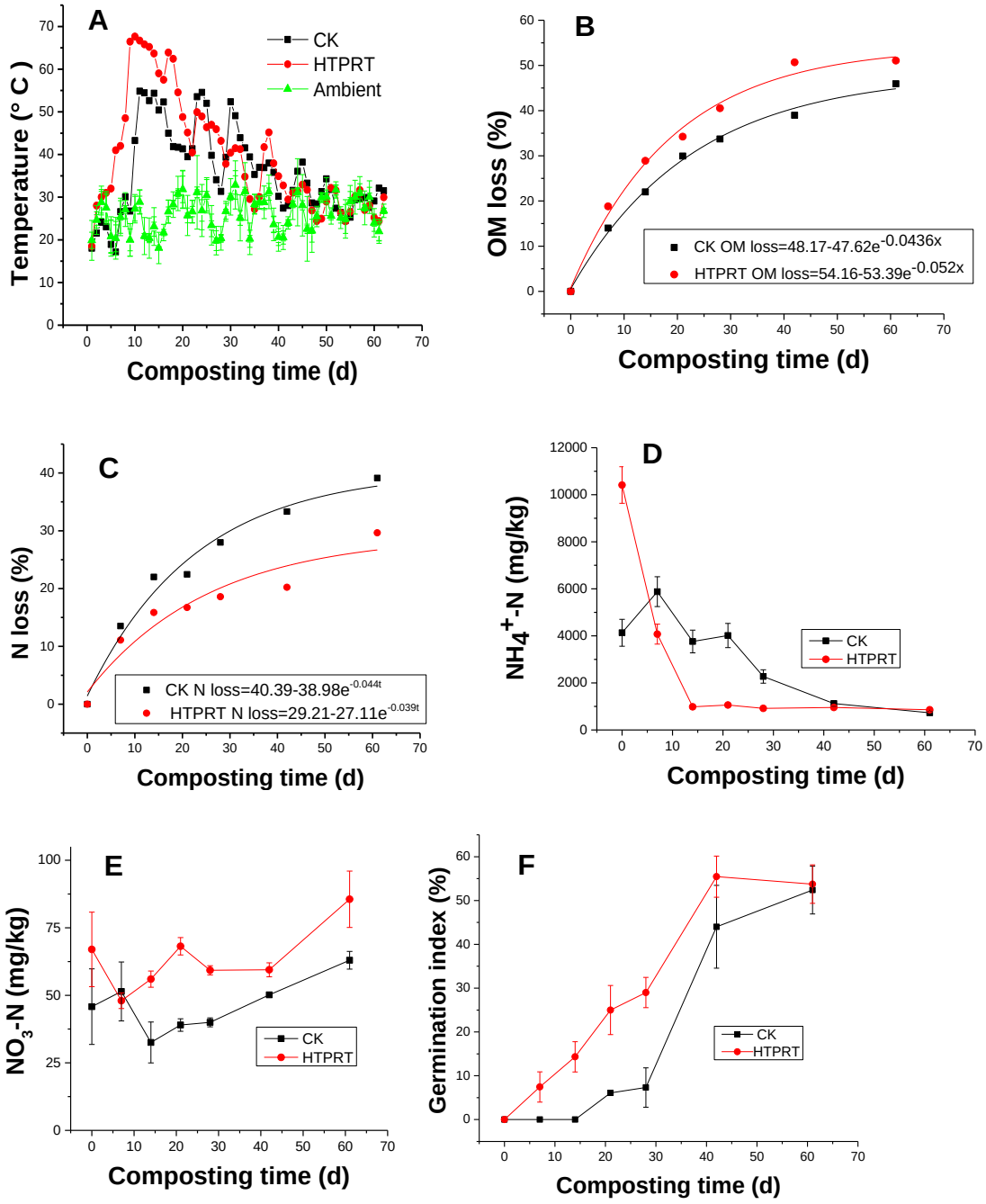


Fig. 2. Evolution of physio-chemical properties during aerobic composting of chicken manure and rice straw with or without HTRPT: a) Temperature; b) OM loss; c) N loss; d) NH_4^+-N; e) NO_3^--N; and f) Germination index

The pH value of the compost with the HTPRT at the start of subsequent composting (Day 0) was approximately 2.2 units lower than the CK compost. This pH difference was attributed to the presence of organic acids formed normally during the HTPRT process of lignocellulosic residues (Nakhshiniev *et al.* 2014). Nevertheless, the pH in the compost from the two treatments turned from acidic to weakly alkaline. This could be explained both by decomposition of organic acids by microorganisms as well as by the release of ammonia from mineralization of organic nitrogen (Fan *et al.* 2017).

The loss of organic matter due to degradation and volatilization or leaching will result in an increase proportion of ash, which does not break down during the composting process (Jindo *et al.* 2016). The initial ash content in the CK and HTPRT was observed as 41.2% and 38.8%, respectively. The change in ash content followed an increasing trend and recorded values of 56.6% and 63.8% for the final composting products from CK and HTPRT (Table 2), respectively. The increased content of the ash in the final product of the HTPRT corresponded to the higher organic carbon mineralization during composting.

The HTPRT exerted a slight effect on the organic carbon degradation dynamics (Fig. 2b). The OM losses followed a first-order kinetic model, as generally found in composting experiments (Sánchez-García *et al.* 2015). The HTPRT piles showed a higher maximum OM loss (a: 54.2% in HTPRT *vs.* 48.2% in CK) and higher rate constant (k: 0.052 day^{-1} in HTPRT *vs.* 0.0436 day^{-1} in CK) and mineralization rate (a × k: 2.8% in HTPRT *vs.* 2.1% OM day^{-1} in CK). The reason behind the stimulation of the OM mineralization in the HTPRT piles might be related to the decreased bulk density as mentioned above.

Compared to OM loss, the HTPRT exerted more pronounced effects on TN loss during composting (Fig. 2c). The HTPRT piles showed a lower maximum N loss (a: 27.1% *vs.* 39.0%) and rate constant (k: 0.039 *vs.* 0.044 day^{-1}). The lower N loss caused by HTPRT suggested a more rapid degradation of organic matter and greater nitrogen retention (Liu *et al.* 2017). These results agreed with the previous studies that the hyperthermophilic composting decreased the total N loss during the composting of cattle manure (Yamada *et al.* 2007).

The evolution pattern of NH_4^+ in the HTPRT piles differed from that in CK (Fig. 2d). In the CK system, there was an increase in the concentration of NH_4^+ during the initial stage of the composting, reflecting rapid OM mineralization. However, in the HTPRT pile, the NH_4^+ concentration showed a downward trend from the beginning of the composting process, and the peak concentration of NH_4^+ appeared on day 1. These results suggested an enhancement of the ammonification process associated to a high mineralization rate during the pretreatment process. Previous studies showed that the amino acid in the compost raw materials was increased by 10.6% (Cao *et al.* 2019). This might be due to the breakdown of protein in the substrate in the hyperthermal pretreatment (Eskicioglu *et al.* 2017). In addition, the nitrification process was also promoted by HTPRT, such that a remarkably higher concentration of NO_3^- was recorded during the maturity stage of composting (Fig. 2e). This might have been due to the better aeration conditions given by HTPRT that enhanced the nitrification process driven by aerobic nitrifying bacteria (Zhang and Sun 2014).

The evolution of maturity as indicated by Germination indices (GI) during the composting process is presented in Fig. 2f. The GI above 50% is defined as a quality parameter for composts produced from manures and slurries (Bernal *et al.* 1998). According to the value of the parameter, both piles achieved a suitable degree of maturity at the end of the composting process. In the first two weeks, the GI values in both piles were quite low (0% in CK *vs.* 14.3% in HTPRT), which could have been due to the high concentrations of ammonium and organic acids (Liu *et al.* 2011). However, HTPRT-treated piles were stabilized and detoxified faster than the control piles. The GI increased to 55% on day 42 and stayed around that value afterwards in the HTPRT piles, whereas the control piles needed an additional 18 days of composting to reach the same degree of maturity.

Table 3. Aerobic Microbial Populations During Composting in Different Systems

Composting Time (d)	Mesophilic		Mesophilic		Mesophilic		Thermophilic		Thermophilic	
	Bacteria (× 10^9 CFU/g)		Fungi (× 10^3 CFU/g)		Actinomycetes (× 10^6 CFU/g		Bacteria (× 10^6 CFU/g)		Actinomycetes (× 10^4 CFU/g)	
	CK	HTPRT	CK	HTPRT	CK	HTPRT	CK	HTPRT	CK	HTPRT
0	5.11b	/	5.21a	/	0.7b	/	0.22c	/	5.88ab	3.78c
7	7.81b	4.12b	3.22a	1.36a	2.72a	0.84c	5.71ab	/	4.33b	2.52c
14	42.38a	3.19b	4.69a	0.18b	0.16b	0.34c	13.26a	5.68c	10.58a	72.28a
21	4.64b	52.7a	/	2.03a	0.19b	4.06ab	1.06b	129.49a	27.03a	51.73ab
28	15.6ab	20.49a	/	/	0.16b	2.4b	4.47ab	47.97b	5.17ab	30.01ab
42	0.02c	2.89b	/	/	0.92b	6.73ab	0.8bc	54.51ab	2.02b	32.02ab

Impact of HTPRT on Evolution of Culturable Microorganisms

The mesophilic microorganisms were undetectable and only thermophilic actinomycetes were detected in the feedstocks after HTPRT according to the plate counts (Table 3), suggesting that the number of OTUs from the 16S rDNA pyrosequencing analysis might be overestimated in the initial stage of subsequent composting. However, as the composting progressed, both the mesophilic and thermophilic microbiota were recovered in the HTPRT piles.

The thermophilic bacteria and actinobacteria reached the highest counts at the thermophilic stage. More specifically, thermophilic bacterial and actinobacterial counts were greater in the HTPRT than the control pile at the thermophilic and maturity stages, suggesting that several species of thermophilic bacteria were recovered at the thermophilic stages and were well adapted to the process conditions (Gannes *et al.* 2013b). Compared to prokaryotic microbiota, mesophilic fungi existed only at the early stage throughout the whole process, and no culturable thermophilic fungi were detected. This might be because very few thermophilic fungi are culturable by classical culture-based methods (Langarica-Fuentes *et al.* 2014).

In general, there was no difference in the microbial evolution throughout composting to what has been reported elsewhere: more microorganisms were detected at the bio-oxidative phase, and a decrease in counts around the thermophilic phases occurred (Steger *et al.* 2007; Partanen *et al.* 2010; Xi *et al.* 2015). However, the extent to which microbial counts, particularly the thermophilic counts, decreased in the HTPRT piles at the maturity stages was much lower.

Once recovered from the HTPRT reactor, composting microbiota in the subsequent composting began to grow faster. These results suggested a growth stimulation of the composting thermophiles that explained the quick activation and maintenance of high temperature in the HTPRT piles (Fig. 2a). In fact, due to hydrothermal reaction, more easily degradable compounds could have been initially released and made available to microorganisms, which in turn would be able to grow and release metabolic heat to produce higher temperatures in the pile (Ding *et al.* 2017; Eskicioglu *et al.* 2017).

Impact of HTPRT on Microbial Community

The composition of bacterial 16S rRNA sequences was determined *via* high throughout sequencing. *Firmicutes*, *Actinobacteria*, *Proteobacteria*, and *Bacteroidetes* accounted for most of the sequences and were considered the dominant phyla (Fig. 3). It has been demonstrated that DNA can be degraded into smaller fragments during heat treatment (Rice and Doty 1957). Surprisingly in the present study, compared to CK0, the community structure of H0 did not change remarkably (Fig. 3), indicating that the heating process did not damage the integrity of DNA in the composting materials. However, an earlier study showed that the bacterial community in the composting materials containing cow dung and sawdust differed much between the composting processes with and without HTPRT (100 °C, 2 h) (Yamada *et al.* 2008). The difference between the two studies might be due to different temperature, duration, and size of the DNA fragment to be amplified (Musto *et al.* 2014).

The temperature in the heating process of this study was lower than the critical temperature value for DNA degradation, which is between 88 °C to 100 °C. The size of the DNA fragment was much smaller in this study, and smaller DNA fragments are easier to be amplified (Sakalar *et al.* 2012; Musto *et al.* 2014).

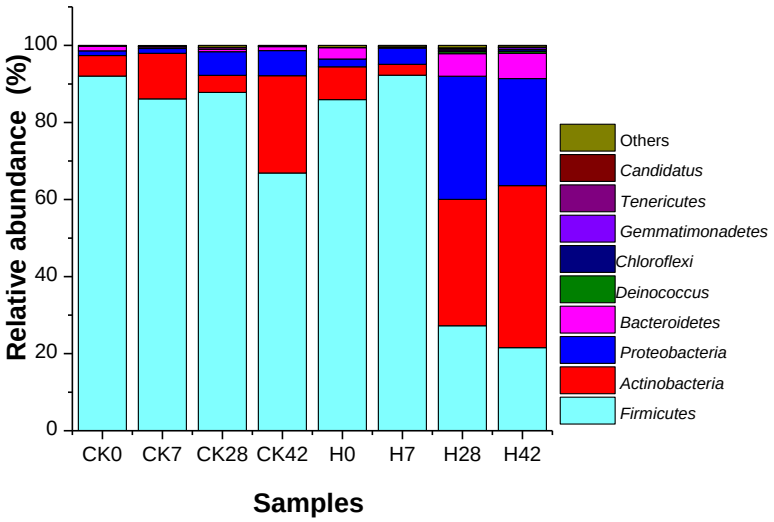


Fig. 3. Taxonomic classification at the phylum level of predominant 16S rRNA gene sequences (relative abundance at top 20) in compost samples during aerobic composting of chicken manure and rice straw with or without HTRPT. The labels CK0, CK7, CK28, CK42 represent samples collected at days 0, 7, 28, and 42 from composts without HTPRT and H0, H7, H28, H42 represent samples collected at days 0, 7, 28, and 42 from composts with HTPRT. The same labeling system is used in subsequent figures.

Species succession occurred over time in both composts. *Firmicutes* was the most abundant bacteria during the whole CK composting process (Fig. 3). In the initial stage (D0), *Lactobacillus* (CK 42% *vs.* HTPRT 28%) was the most abundant bacteria in both piles. As composting progressed, *Ureibacillus* (22.68%, phylum *Firmicutes*) and *Ammoniibacillus* (14.08%, phylum *Firmicutes*) were the predominant species in the thermophilic phase of the HTPRT pile, while the curing phase was dominated by *Thermobifida* (12.75%, phylum *Firmicutes*), *Saccharomonospora* (11.81%, phylum *Actinobacteria*), and *Georgenia* (8.9%, phylum *Actinobacteria*). However, *Bacillus* was the predominant species during the thermophilic (26.30%) and curing (28.56%) stages of CK (Fig. 4). The authors' results revealed that the HTPRT increased the relative abundance of *Actinobacteria* but decreased the level of *Firmicute*s compared to the traditional composting (Fig. 3). These results were consistent with the bacterial community in hyper-thermal compost derived from industrial waste materials in the literature, which state that the predominant species present are *Saccharopolyspora* (32.9%) and *Georgenia* (15.7%). To the authors' knowledge, these genera have not been reported to be predominant in any other composts obtained by traditional composting (Lv *et al.* 2015; Zhang *et al.* 2016a), and little is known of their functions and roles in the HTPRT composting process. Because *Actinobacteria* is considered an important lignocellulose degrader in composts (Ryckeboer *et al.* 2003; Steger *et al.* 2007), a higher abundance of *Actinobacteria* in HTPRT piles suggests that a bacterial community with a more stable structure could be developed in the later stage (Zhang *et al.* 2016b). Enhancing the populations of *Actinobacteria* could speed up the degradation of macromolecules, such as cellulose, hemicellulose, and lignin, thus improving the composting efficiency. However, *Firmicutes* (74%) was predominant in sewage hyper-thermal compost samples (Tashiro *et al.* 2016). These results suggested that a distinct bacterial community structure was formed in the HTPRT process. Additionally, even with HTPRT, the predominant phyla were diverse and specific in composts with different raw materials (Tashiro *et al.* 2016).

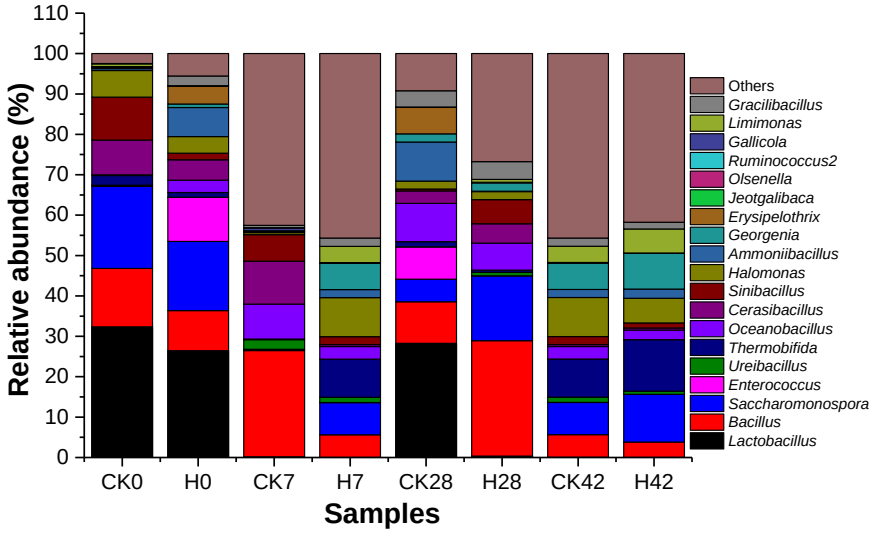


Fig. 4. Taxonomic classification at the genus level of predominant 16S rRNA gene sequences (relative abundance at top 20) in compost samples collected at days 0, 7, 28, and 42 during aerobic composting of chicken manure and rice straw with or without HTRPT

Bacterial Community and its Association with Physio-chemical Parameters

The CCA was used to analyze the relationship between environmental parameters (temperature, TOC, TN, pH, NH_4^+-N, or NO_3^--N) and bacterial community (Fig. 5a). The first two axes of the CCA explained 53.2% and 23.6% of the variation in the species data, respectively. As shown in Fig. 5a, the CCA plot grouped eight compost samples into four distinct clusters. Compost samples collected at day 0 (CK0, H0) and day 7 for the CK were separated from those collected at day 28 and day 42, suggesting that the bacterial community profiles in the raw materials and CK samples collected at the initiation stage were similar. As the composting progressed, samples collected from HPC on day 7 and from CK on day 28 were clustered together. The results suggested an important role of temperature for shaping the microbial structures in the CK and HTPRT piles, and the effect was more noticeable in HTPRT. Notably, H28 and H42 samples were clustered together, and they were separate from the samples of CK42, which suggested that composts in HTPRT became stable after 28 days of composting and reached maturity over 14 days in advance than that of CK. Similar results were reported by Yu *et al.* (2018), stating that sewage sludge from hyperthermophilic composting became mature after 24 days of composting, while composts collected on day 48 in traditional composting were still immature.

A specific CCA analysis was performed on the relationship between bacterial community and the above selected 6 environmental factors in the HTPRT (Fig. 5b). The first two CCA axes explained 5 1.8% and 23.1% of the variation in the species-environment relationship. In this plot, the 20 most abundant OTUs identified at the genus level were divided into three groups throughout the HTPRT composting. Group 1 was positively associated with TOC, NH_4^+-N, and NO_3^--N, suggesting that the microbial community structure was likely to be affected by the mineralization rate of organic carbon and nitrogen, while remarkable relationships existed between the temperature and Group 2. Thermotolerant microorganisms are believed to play a major role in organic matter degradation in composts. Kanazawa *et al.* (2008) reported that the metabolic heat released from the bio-fermentation of organic wastes raises the processing temperature sufficiently

high at 70 °C to 100 °C to support thermophilic bacteria in HTPRT, such as *Ureibacillus* and *Ammoniibacillus*, which belong to the order of *Bacilli* in the thermophilic stage of composting. However, when the temperature of the pile cooled down, the moderately thermophilic bacteria, such as *Saccharomonospora* and *Thermobifida*, which both belong to the order of *Actinomycetales*, became the predominant species. This could be further evidenced by the enhanced populations of these thermophilic microorganisms in the thermophilic and cooling stages of the HTPRT pile. Group 3 was related to TN and NH_4^+-N, suggesting their roles in the organic N formation during the humification process. This suggested that Group 3 indicated the maturity of HTPRT. Early studies reported the reappearance of *Saccharomonospora* in the maturation stage, suggesting this group of microorganisms may be a potential indicator of compost maturity (Steger *et al.* 2007). In addition, due to the high salt contents in animal manures, an enhanced population of halotolerant *Halomonas* and *Gracilibacillus* may have also contributed to the shortened length of animal waste composting (Tang *et al.* 2011).

Notably, the authors previously isolated extreme thermophiles growing at > 80 °C in the hyper-thermal composting process, such as *Rhodothermus* sp. CGMCC 7.246 and *Thermus* CGMCC 7.247. However, these extreme thermophiles were not dominant in the compost as revealed by Illumina pyrosequencing in the present study, while the *Thermus* was predicted to be the dominant genus responsible for the hyperthermophilic composting of sewage sludge (Yu *et al.* 2018). The difference in the dominant species may be mainly be due to the different composting techniques applied. In their study, a period of 9 days for the hyperthermophilic phase (> 80 °C) was recorded, while only 4 h of hyperthermophilic pretreatment of the raw materials was applied in the present study.

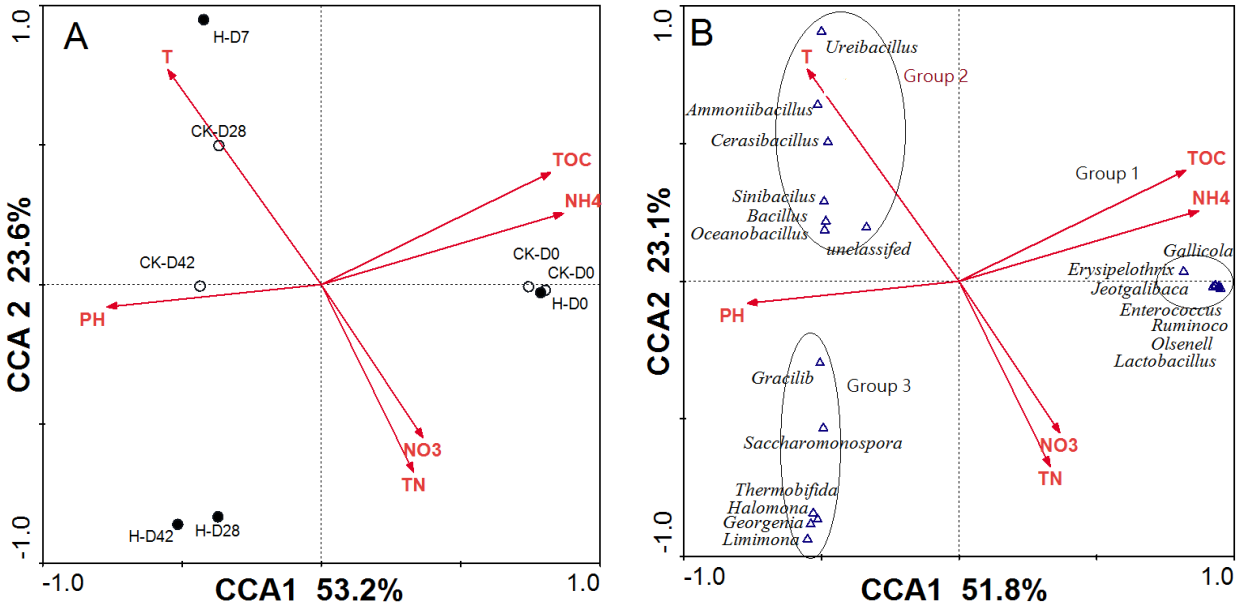
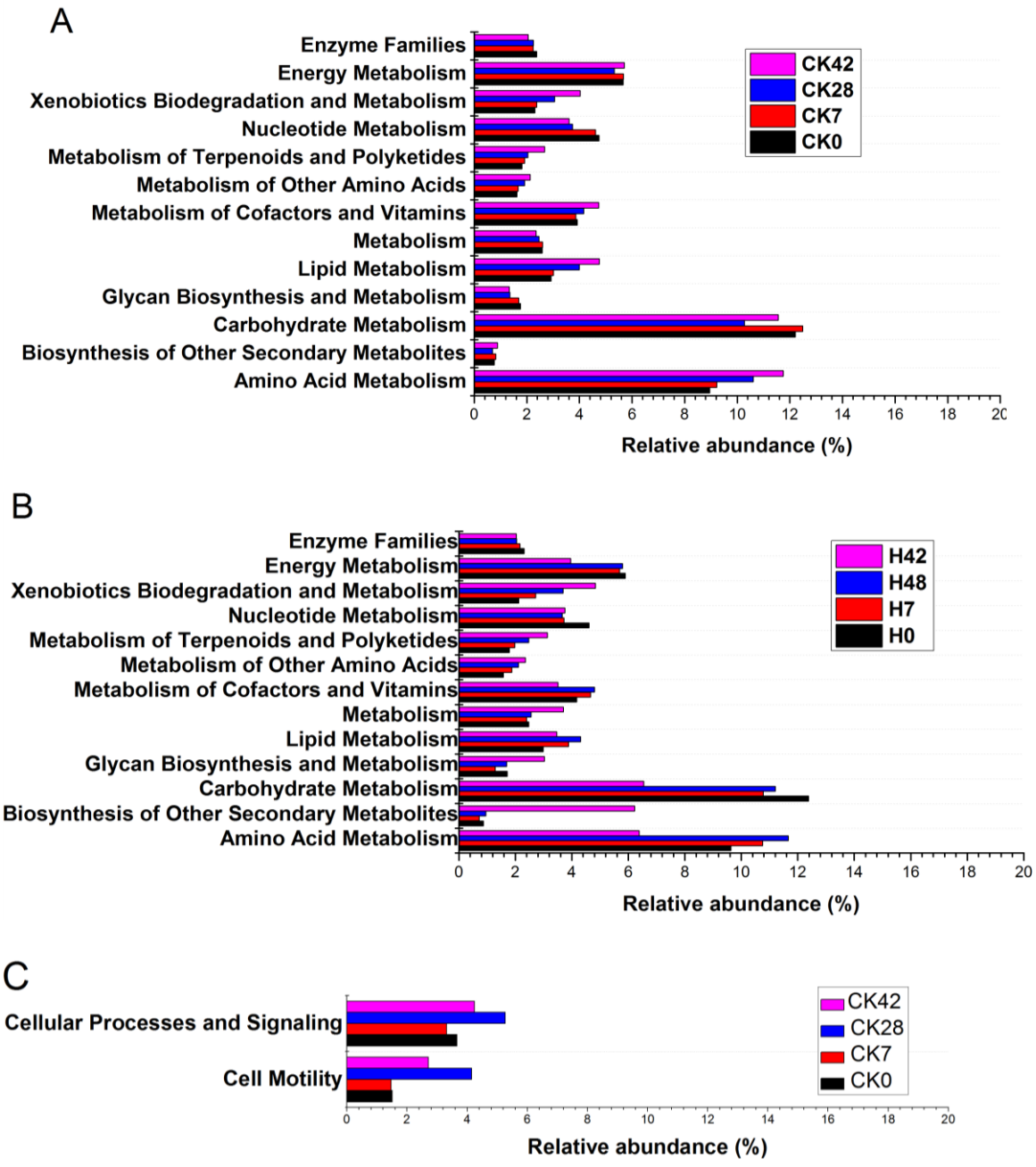


Fig. 5. (a) Canonical correspondence analysis (CCA) between microbial community and physio-chemical parameters for aerobic composting of chicken manure and rice straw with or without HTRPT; (b) CCA of the correlation between microbial community and physio-chemical parameters for HTPRT piles

Bacterial Function Prediction

PICRUSt is a highly regarded bioinformatics tool that allows prediction of functional profiles based on phylogenetic composition of communities using databases KEGG and COG (Samaddar *et al.* 2019). The bacterial metabolic properties during composting in the two piles were predicted using PICRUSt based on 16s rRNA homology and conserved feature of functional contribution (Fig. 6). There were four functional groups of the genes, including metabolism (Fig. 6A, 6B) (51.6% to 57.6%), environmental information processing (Fig. 6E, 6F) (15.6% to 22.5%), genetic information processing (Fig. 6G, 6H) (15.6% to 22.5%) (16.1% to 25.7%), and cellular processes (Fig. 6C, 6D) (4.9% to 9.7%) (Fig. 6).



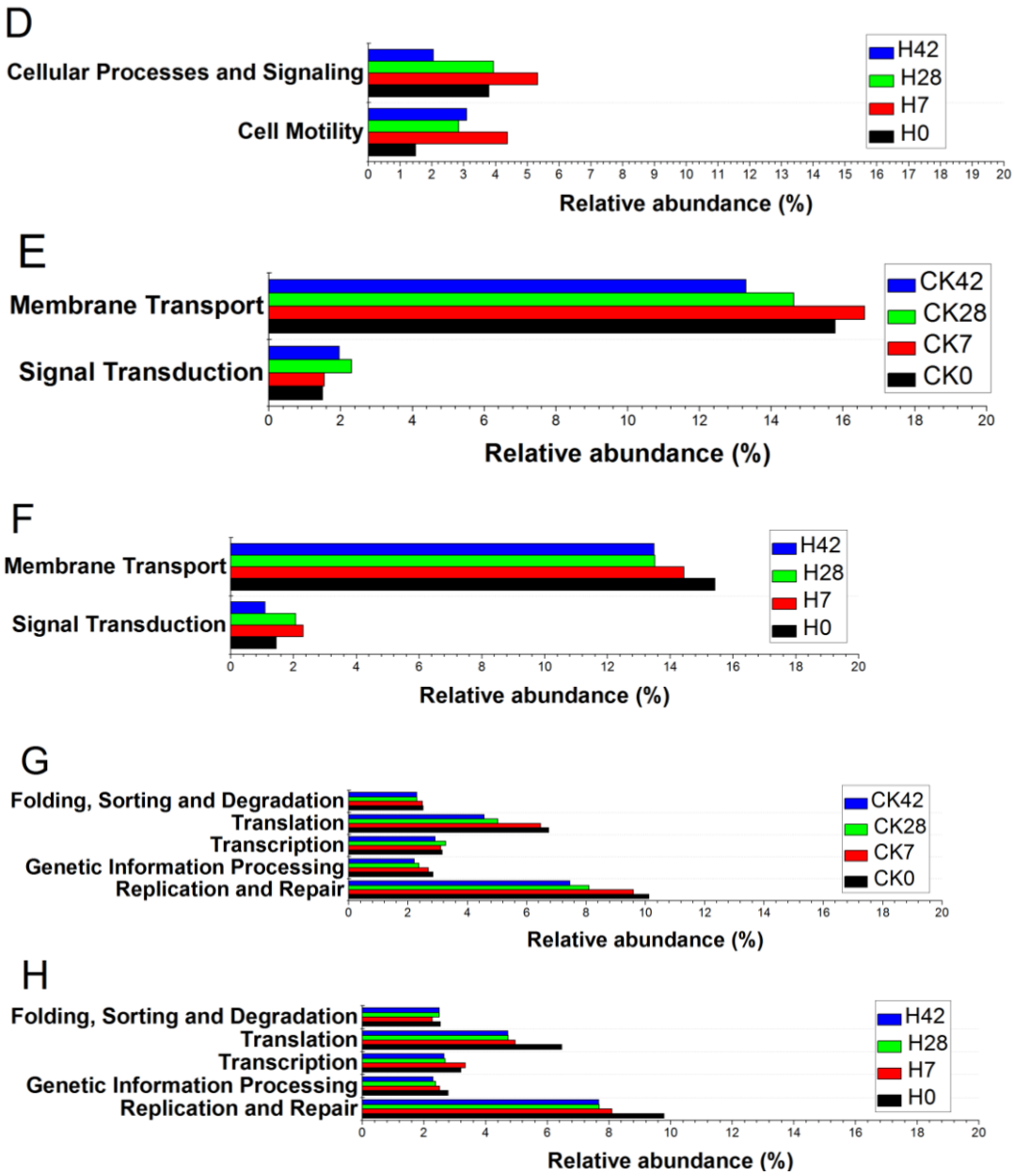


Fig. 6. PICRUSt function prediction based on KEGG database

The most frequently occurring type in the metabolism category was carbohydrate metabolism (6.5% to 12.3%), followed by amino acid metabolism (6.4% to 11.7%), energy metabolism (3.9% to 5.9%), and nucleotide metabolism (3.6% to 4.7%). Bacterial members originating from composts pretreated with HTPRT were characterized by higher abundance of genes encoding enzymes related to amino acid metabolism, carbohydrate metabolism, and lipid metabolism compared to composts without HTPRT in the initial and thermophilic stages of composting (day 0 and day 7). These results are in line with the previous observations where common enzymatic activities like dehydrogenase, protease were significantly higher in hyperthermophilic composting compared to traditional composting (Xiao *et al.* 2009). The genera involved in carbohydrate and amino acid metabolism remarkably increased during the first 28 days in the HTPRT pile and dramatically decreased at day 42. However, the sequences related to carbohydrate and

amino acid metabolism showed a continuous increasing trend in CK. These results are supported by the observations where faster degradation of organic matter recorded in the HTPRT in the active stage of composting process. The results observed imply that the HTPRT pile reached a stabilization period (maturity stage) in advance because biodegradable carbohydrate and amino acids rapidly decreased in the sludge during the thermophilic period (Wu *et al.* 2017; Wang *et al.* 2018).

CONCLUSIONS

1. The authors' results demonstrated that the composting process was remarkably enhanced by hyperthermophilic pretreatment (HTPRT) at 85 °C for 4 h.
2. The HTPRT decreased bulk density, facilitated the solubilization of complex organic components into small ones, accelerated OM stabilization, and shortened the composting process 18 days.
3. The HTPRT favored N mineralization and enhanced N retention.
4. Distinct microbial communities were formed in the thermophilic and maturity phases dominated by thermotolerant *Bacilli* and *Actinomycetales*, indicating the bacteria were mainly responsible for the degradation of organic matter. The results of bioinformatics analysis using PICRUSt demonstrated that poultry wastes pretreated with HTPRT were characterized by abundance of genes encoding enzymes contributing to amino acid metabolism, carbohydrate metabolism and lipid metabolism in the active stage of composting.

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