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ORIGINAL ARTICLE

Inoculation of cattle manure with microbial agents increases efficiency and promotes maturity in composting

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Abstract

In this study, fresh cattle manure was mixed with rice straw at a ratio of 10:1 in fresh weight and then composted in a self-built, aerated static composting box, whose dimension was 1 m × 0.8 m × 0.8 m with a volume of approximately 0.6 m^3. To deal with the inconvenient and time-consuming problem of multiple stage inoculation, a single, one-time inoculation agent containing diverse microorganisms that are active at both the initial heating and thermophilic phases was developed. A total of 12 from 42 strains isolated from the none-inoculated Experiment 1 composting system were selected as microorganismal agents in Experiment 2 according to their species, prevalence and cultural temperature. 200 mL of each microorganism enrichment broth was mixed to the inoculation group at the beginning of composting. A total of 2400 mL of sterilize distilled water was added to the control group. The parameters of temperature, moisture, pH, C/N ratio, organic matter degradation, and germination index were investigated for both inoculation and control composting groups. Results showed that inoculation did not significantly shorten composting time. However, the pile temperature was increased with the maximum temperatures of 64.6 °C and 60.3 °C for the inoculation and control groups, respectively. The degradation of organic matter was accelerated ($P < 0.05$), and significantly higher GI value ($P < 0.05$) indicated that the maturity was promoted by the inoculation microorganism. This suggests that the final composting product would provide value as alternative source of nutrients for plants. Conclusively, we suggested a multiple microorganism inoculation method to increase the efficiency and promote maturity in cattle manure composting.

Keywords Composting · Multiple microorganism · Inoculation · Cattle manure

Introduction

Animal production in China has achieved considerable progress, contributing to 46% of the total agriculture output value of the country. However, this fast expansion of animal production has led to an increase in environmental pollution (Tan and Yin 2016). More than 2.56 billion tons of cattle manure was produced in China in 2015, a result generated by the rapid development of the livestock industry according to the report by the National Bureau of Statistics of China (2016). This rapid development has increased the cattle population to over 108 million heads. Such a significant increase in waste production necessitates urgent management using appropriate disposal practices, or else it could bring about large-scale environmental pollution, especially for soil and water. Composting is a common and effective method for treating cattle manure and the product can be used as organic fertilizer (Asano et al. 2010; Larney and Hao 2007; Sun et al. 2016; Xu et al. 2016). During the process, bacteria, fungi and other microorganisms, including micro arthropods, break down organic material to stable and usable organic substances called compost (Bernal et al. 2009). The degradation process always occurs in nature; however, many artificial measures have been developed to accelerate the biodegradability of the indigenous microbial

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community. Inoculation is a human-induced measure that could help increase the initial microbial population, enhance the amount of viable microbial communities, generate the desired enzymes, and thus significantly enhance the composting process (Xi et al. 2007).

Jiang et al. (2015) reported that adding 1% NTB (ammonifiers, nitrobacteria, and *Azotobacter*) agent at the initial start of composting could reduce N loss and could effectively promote composting maturity. Nakasaki et al. (2013) demonstrated that the yeast strain *Pichia kudriavzevii* RB1 affected the early stages of composting prior to the thermophilic stage and accelerated the overall composting process. Xi et al. (2012) also found that inoculating microbes also increased the molecular weight, humic- and fulvic-like compound content, as well as humification degree of the composting products in the order of initial-stage < two-stage < multi-stage inoculations during the municipal solid waste and dry grass composting process. Furthermore, Xi et al. (2015) reported that the multi-stage inoculation method extended the high temperature period and improved the community diversity of bacteria and fungi. These multi-staged inoculations reduced the competition between inoculations and indigenous microbes, enhancing the growth of inoculated microorganisms. Similar results were also found by Zhao et al. (2016) using agricultural waste compost, observing that inoculation in different stages of composting could distinctly accelerate the degradation and improve the actinobacteria community diversity, particularly in the cooling stage of composting.

These previous studies indicated that the inoculated microorganisms would increase degradation of organic matter above the capabilities of the indigenous microorganisms, and that it is necessary to add various inoculations across multiple stages of the composting process. However, it is inconvenient and time consuming for livestock farmers to divide up manure in different stages of composting and mix the relevant inoculations of each pile at each individual stage. To deal with these issues, a single, one-time inoculation agent containing diverse microorganisms that are active at both the initial heating and thermophilic phases is needed. In this study, a mixture of 12 microorganism agents, including two strains of *Bacillus licheniformis*, three strains of *Bacillus megaterium*, and a single strain each of *Bacillus thuringiensis*, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Cellulosimicrobium funkei*, *Cellulomonas* sp., *Thermomonospora* sp. and *Streptomyces* sp. were isolated from natural composting piles and inoculated at the initial stage of the composting process. Differences between control and inoculated piles were evaluated. The overarching goals of this study were to compare the influence of a multiple species inoculant on cattle manure composting.

Materials and methods

Composting materials and reactor

Cattle manure and rice straw collected from a cattle farm and local residents in Dalian, China, were used as the raw materials for the present study of aerobic composting. Rice straw, used to adjust the water content and suitable C/N for composting, was mixed with fresh cattle manure at a ratio of 1:10 in fresh weight and then composted in a self-built, aerated static composting box. The dimensions of each bin were 1 m × 0.8 m × 0.8 m with a volume of approximately 0.6 m^3. The installation of the reactor is shown in Fig. 1. The outer wall and the top of the reactor were insulated with common cotton quilts to maintain the thermo-energy

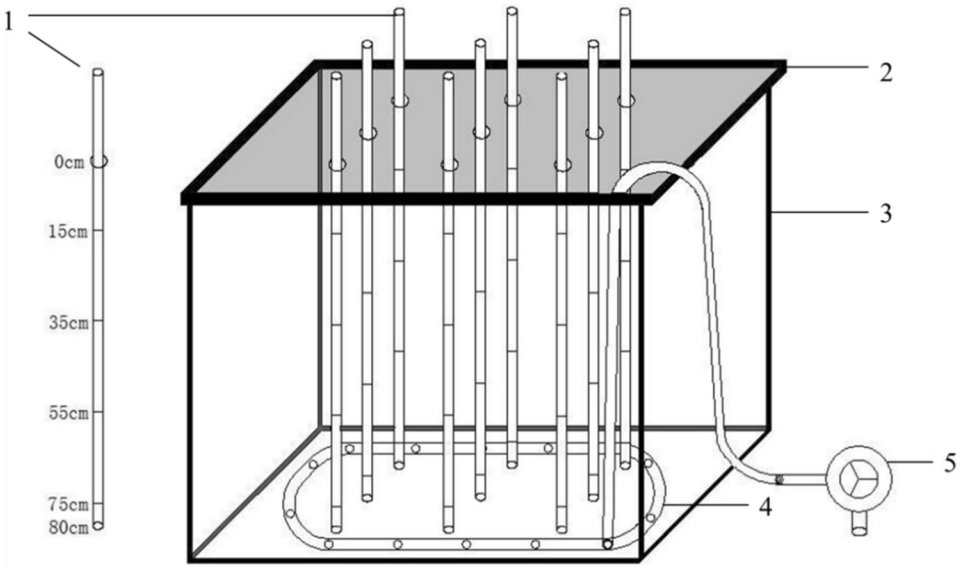

Fig. 1 Compost reactor. (1) bamboo canes with thermocouples; (2) lid; (3) plastic composting box; (4) PVC tubes; (5) air pump

produced during composting. Ventilation was performed by an air pump through PVC tubes buried in the composter.

Experiment 1: isolation of microbes from compost

Design and sampling

The composting was created with approximately 200 kg of fresh cattle manure and 20 kg of rice straw. Once mixed, the water content was adjusted to approximately 65%, requiring about 20 L of water to be added to the mixture. Air was pumped into the bottom of the composter using PVC tubes with an air flow of approximately 6.5 m^3 min^{-1} during composting until the 22nd day, from which the frequency of ventilation was once per day at 1800 for duration of 5 min. Thermocouples bound on bamboo canes were embedded at depths of 15, 35, 55 and 75 cm to monitor temperature changes. Temperatures were measured three times per day, at 09:00, 13:00 and 17:00. Nine points were set for each depth as shown in Fig. 1, and the average temperatures were recorded every day. The ambient air temperature was monitored simultaneously. Piles were turned once on the 10th day. Three samples were collected across multiple days (day 1, 2, 3, 4, 6, 8, 10, 11, 12, 13, 15, 17, 19, 21 and 22) from each of three pile locations and stored at − 20 °C for later analysis. Three grams of each sample was diluted into 27 mL sterilized distilled water, vortexed for 5 min and left at room temperature for 30 min to precipitate the solids. Supernatants were measured for pH using Sartorius PB-10 (Sartorius, Germany). The water content was calculated by drying the samples in an oven at 105 °C and the C/N ratio was detected using a Vario EL III elemental analyzer (Elementar, Germany).

DNA extraction and PCR–DGGE analysis

A total of 12 samples collected on day 1 (22 °C), 2 (39 °C), 4 (54 °C) and 6 (57 °C) were extracted for DNA using the commercial QIAamp DNA Stool Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. To investigate the dominant microorganisms in the initial heating and thermophilic phases, 16S rRNA genes were amplified using the prokaryotic primers 341F/534R (Muyzer et al. 1993). The nucleotide sequences of the primers are as follows: primer 1 (5′-CCTACGGGAGGCAGCAG-3′), primer 2 (5′-ATTACCGCGGCTGCTGG-3′), and primer 3 (5′-CGCCCG CCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGG GCCTACGGGAGGCAGCAG-3′). The PCR amplification was performed as described by Muyzer et al. (1993). Analyses using denaturing gradient gel electrophoresis (DGGE) was carried out by loading successfully amplified PCR products into polyacrylamide (8%) gels with gradients of 30–70% of denaturants (urea/formamide) and run at 60 V for 7 h at 60 °C to separate fragments. After electrophoresis, gels were stained in ethidium bromide and photographed under UV light. DNA from the cut bands were eluted by rinsing for 10 min with Milli-Q water, and used as templates for reamplification with the original primer pair (without GC clamp) (Muyzer et al. 1993). All sequences were analyzed, and the PCR fragments were sequenced by Life Technologies (Shanghai, China).

Microorganism isolation

Those samples collected at day 2 (initial heating phase) and day 6 (thermophilic phase) with a temperature of 39 °C and 57 °C, respectively, were used for microbial isolation. Samples were diluted in PBS to the concentration of 10^{-6}–10^{-8}, and 100 µL of each dilution was streaked onto beef extract peptone (BEP) and Gauserime synthetic (GS) agar (Hopebio, Qingdao, China) and incubated at both 37 °C and 55 °C for detection of bacteria and actinomycetes. After a 48 h incubation, colonies were selected from agar plates and inoculated onto the same broth mediums according to their shape, size and color from which colonies were displayed. Single strains were purified by inoculating and streaking three times, then sequenced for 16 s rDNA to identify the microbial group using the primers 27F (5′-AGAGTTTGA TCCTGGCTCAG-3′) and 1429R (5′-TACGGCTACCTT GTTACGACTT-3′) (Polz and Cavanaugh 1998) under the following PCR conditions: 94 °C for 4 min; 35 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s, followed by a hold at 72 °C for 10 min.

Experiment 2: inoculation of compost

Two groups of cattle manure composting experiments were performed under the same conditions of Experiment 1. The first group is the inoculation, which was conducted with 12 microorganismal agents isolated from Experiment 1. The microorganisms were cultured in BEP broth (Hopebio, Qingdao, China) for 48 h to a concentration of 10^8 CFU/mL, and 200 mL of each enrichment broth was inoculated at the beginning of composting. A total of 2400 mL of sterilized distilled water was added to the control group. The entire composting period lasted 28 days. Piles were turned once every 7 days. Three samples were additionally collected on days 1, 2, 3, 4, 6, 8, 9, 10, 13, 15, 16, 17, 20, 22, 23, 24, 27 and 28 from each of the three locations and stored at − 20 °C for later analysis. The temperature, pH, water content and C/N ratio were measured as described in Experiment 1 (see above). In addition, the percentage of organic matter was detected according to the Chinese standard of NY525-2012 (2012). The concentration of cultivable microorganism was examined by diluting samples in PBS to the concentration of 10^{-6}–10^{-8}, and 100 µL of

each dilution was streaked onto BEP agar (Hopebio, Qingdao, China) and incubated at the pile temperature during the time of sampling. After a 48 h incubation, bacterial colonies were counted. The germination index (GI) and the change of inactivation index of *Escherichia coli* were tested according to the formula (Zucconi et al. 1981):

$$\mathrm{GI}\,(\%) = \frac{\text{seeds germination intreatment}\,(\%) \times \text{root length in treatment}}{\text{seeds germination in control}\,(\%) \times \text{root length in control}} \times 100\%.$$

For the control group, 20 corn seeds purchased from local seeds company were put between two pieces of filter paper soaked with 5 mL leaching liquor at the ratio of 1 g (sample) per 10 mL sterilized distilled water. After a 48 h incubation at 25 °C, the germinated corn seeds were counted and sterilized using distilled water. The number of *E. coli* was counted by Petrifilm *E. coli* count plate (PEC) as described by Matner et al. (1990).

Statistical analyses

The statistical significance of differences between all replicated samples was determined using GraphPad Prism 5 using a one-way ANOVA analysis and *t* test analytical methods. The significant level of differences in the research was set as $P < 0.05$.

Results

Variations in temperature, moisture, pH, and C/N ration during Experiment 1

Figure 2a illustrates the temperature profiles observed during the composting process. Temperature during composting went through three distinct phases, including an initial

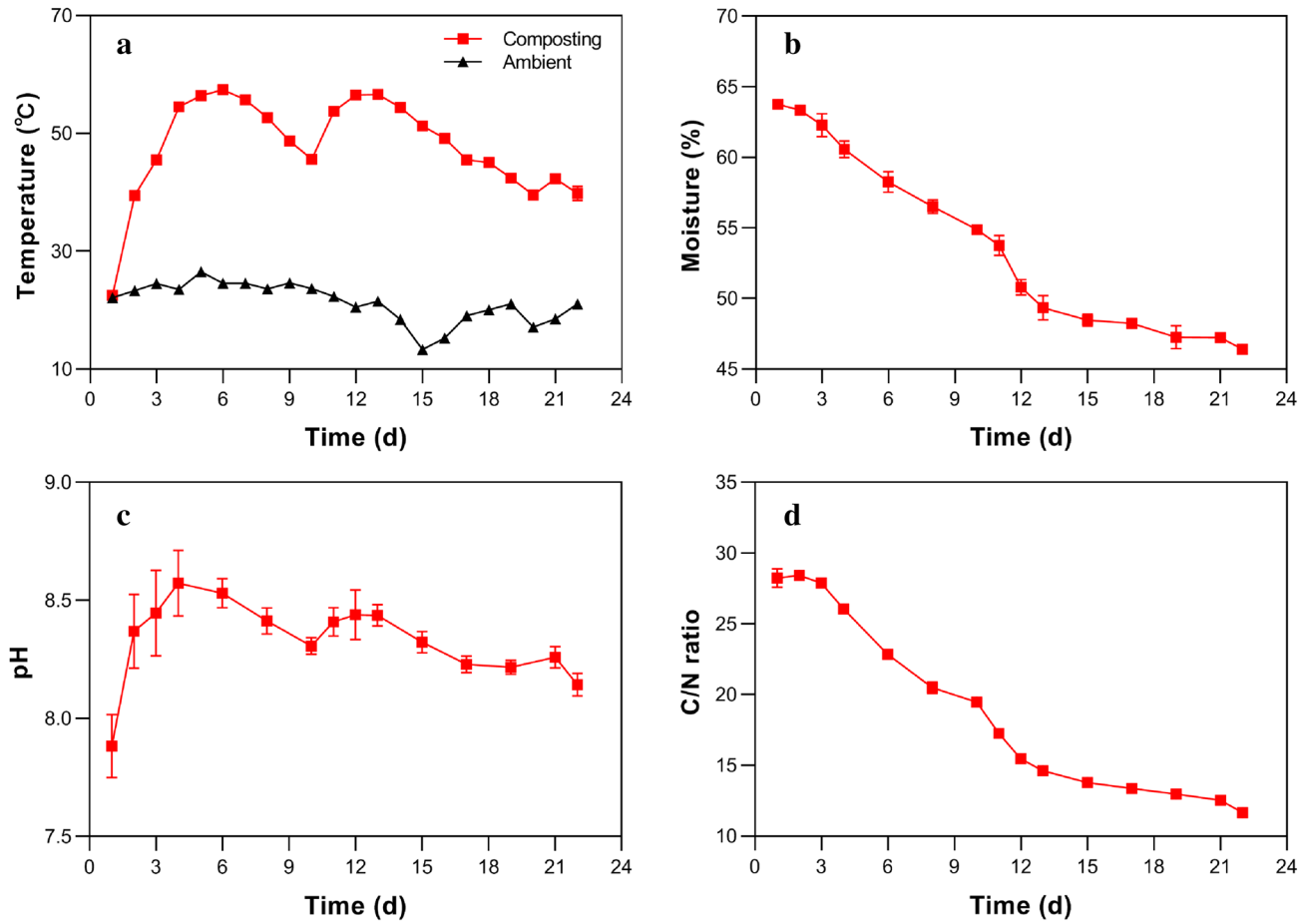

Fig. 2 Changes in **a** temperature; **b** moisture; **c** pH; and **d** C/N ratio during Experiment 1

heating, thermophilic and cooling. Temperature was measured three times a day and nine points for each of four depths as described above. The average temperature ranged from 22.5 to 57.4 °C throughout the entire study period. The maximum temperature obtained in the middle of the pile was 57.9 °C on day 6. The thermophilic phase (> 50 °C) lasted for 5 days for each of the stages and was reached during days 4–8 and 11–15, respectively (Fig. 2a). The temperature of the compost increased again after turning on day 10. The moisture content decreased from 63.8 to 46.4% over the 22 days of composting (Fig. 2b). Our pH values increased rapidly with increasing temperature, reaching peak values of 8.57 at day 4 (Fig. 2c), and changed accordingly with the observed temperature variations. The C/N ratio in Fig. 2d increased during the first 2 days, then decreased until the end of the composting period.

Microbial community structure of Experiment 1

Molecular analysis on the diversity of the microbial communities found in composting cattle manure was performed during the natural degradation process. The DGGE band patterns shown in Fig. 3 illustrate the eukaryotic communities observed from the initial heating and thermophilic phases of Experiment 1. The banding patterns showed a significant difference among the two composting phases. *Bacillus* bacteria were found in both samples, including the initial heating and thermophilic phases. *Bacillus licheniformis*, *Bacillus subtilis* and *Bacillus cereus* were predominant at the initial heating phase, while different strains of *Bacillus licheniformis* and *Bacillus aerius* were detected in thermophilic phases. *Thermoactinomyces*, *Bacillus amyloliquefaciens* and *Streptomyces* were prevalent in the thermophilic phases. *Cellulosimicrobium funkei* and *Cellulomonas* sp. were only present during the thermophilic phases.

Microorganisms gained from Experiment 1

A total of 42 strains were isolated from the composting system. Plates at 37 °C yielded 11 *Bacillus*, 2 *Thermoactinomyces* and 1 *Nocardia* sp., while 18 *Bacillus*, 2 *Cellulomonas*, 2 *Pseudoxanthomonas*, 3 *Thermoactinomyces,* 2 *Streptomyces* and 1 *Micromonospora* sp. were obtained from the plates at 55 °C. *Bacillus, Thermoactinomyces* and *Streptomyces* sp. were the predominant isolated strains, consistent with the results of our DGGE analyses. A total of 12 strains were chosen as microorganism agents for the cattle manure composting experiment according to their species, prevalence and cultural temperature. This included two strains of *Bacillus licheniformis*, three strains of *Bacillus megaterium*, and a single strain of *Bacillus thuringiensis*, *Bacillus subtilis*, *Bacillus amyloliquefaciens* and *Thermomonospora* sp., which were detected from both 37 and 55 °C plates. A single strain of *Cellulosimicrobium funkei*, *Cellulomonas* sp. and *Streptomyces* sp, which were only isolated from 55 °C plates were also selected as part of microorganism agents.

Effect of Experiment 2: inoculation of compost

The temperature in the two treatments (inoculation and control) reached the thermophilic phase (i.e., more than 50 °C) at days 2 and 3, respectively (Fig. 4a). The maximum temperatures in all treatments was obtained after 4 or 5 days, with the average temperature being between 64.6 and 60.3 °C for the inoculation and control groups, respectively. The temperature curves fluctuated according to turning of the piles in both groups. Overall, the thermophilic stage lasted for 23 days in both treatments except day 22 of the inoculation group, where the average temperature was 49.4 °C. In the beginning of the composting experiment, the inoculation treatment group rapidly reached a temperature

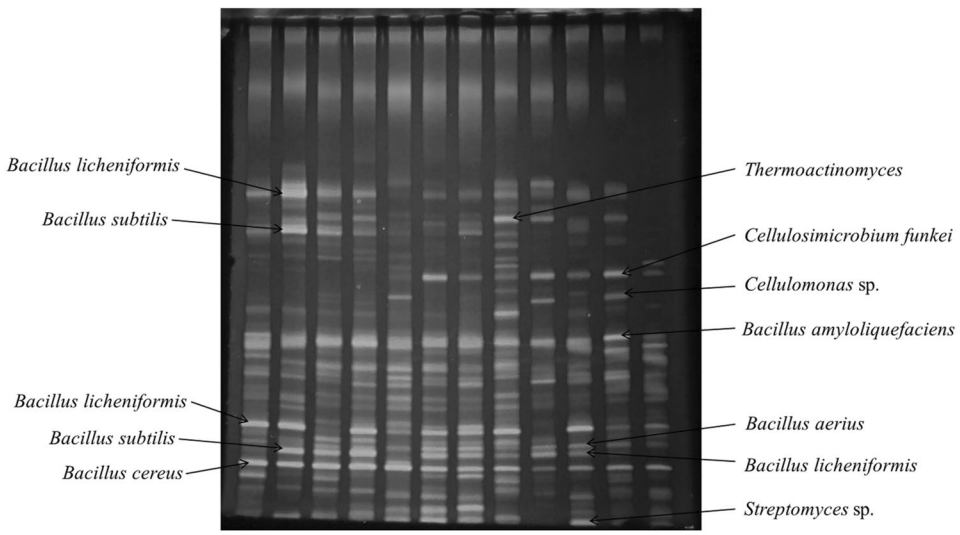

Fig. 3 DGGE profile of microbial communities in cattle manure compost in Experiment 1

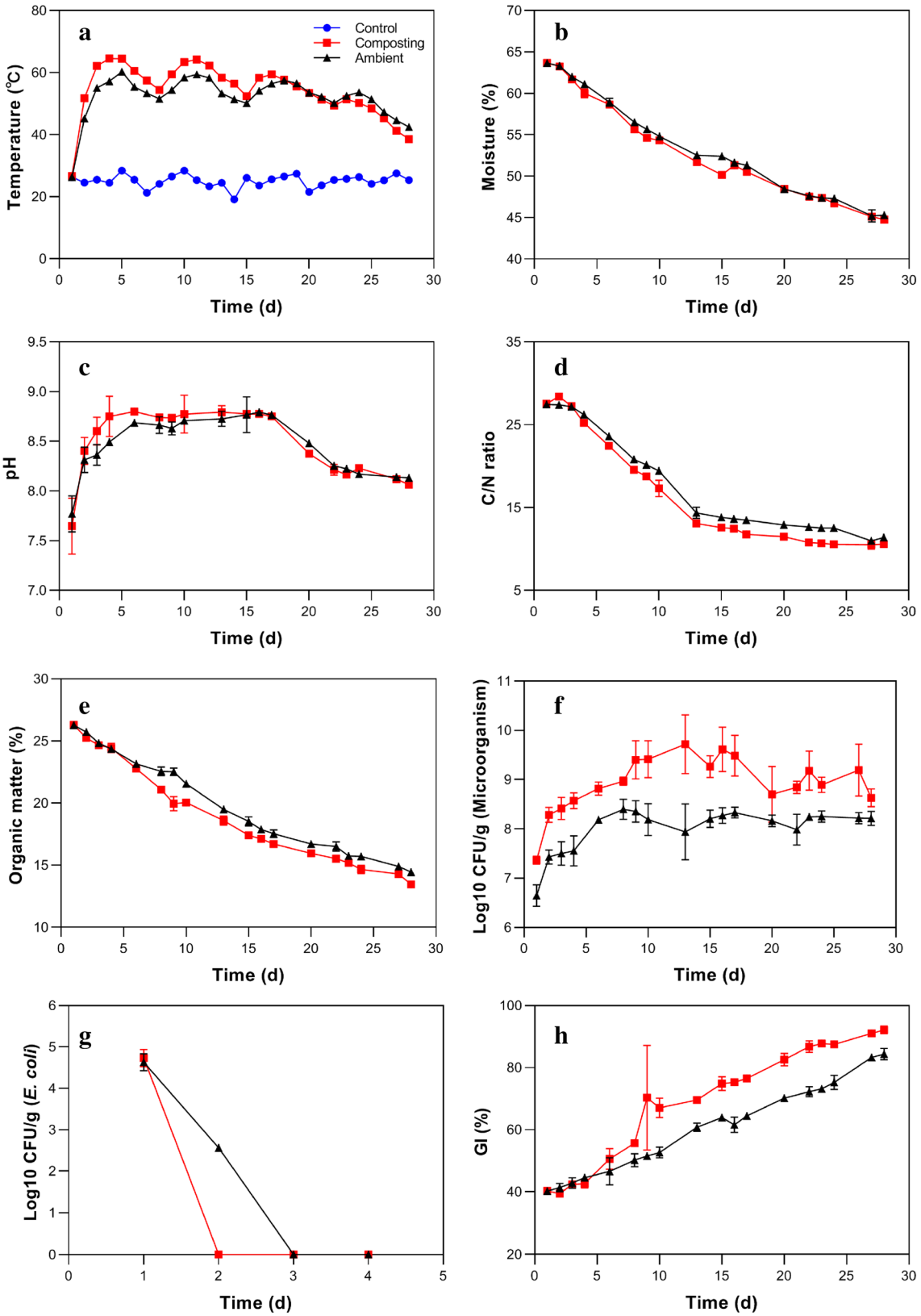

Fig. 4 Changes in **a** temperature; **b** moisture; **c** pH; **d** C/N ratio; **e** organic matter; **f** microorganisms; **g** *Escherichia coli*; and **h** GI (germination index) during Experiment 2. Bacterial inoculations devised in Experiment 1 were used for the inoculation group

of 51.8 °C on day 2. The moisture of each pile continuously decreased from approximately 64% at the beginning of composting to 46% at completion (Fig. 4b). Changes in pH between the two composting groups are shown in Fig. 4c. The pH increased rapidly with increasing temperatures and reached 8.75 on day 4, lasting for 14 days until day 17, with the peak being on day 6 (pH 8.8) and then decreasing to 8.06 at the end of the composting period. Initial C/N ratios were 27.5 in both groups (Fig. 4d). In the final compost, C/N ratios dropped to 10.6 and 11.4 in the inoculation and control groups, respectively. Although a small peak was observed in the inoculation group at day 2, the C/N ratio decreased rapidly for both groups and with similar trends until the end of the composting period. The concentrations of OM in the inoculation group was slightly lower than that in the control group, with significant differences ($P < 0.05$) from day 6 to the end of the composting period (Fig. 4e). The only exception to this was on day 13, 24 and 28. Growth curves of the microorganisms during composting of the inoculation and control groups are shown in Fig. 4f. The cell density of microorganisms in both groups increased at the initiation of composting, then decreased to a final concentration level of 10^8 CFU/g (colony forming unit/gram). In contrast, cells of *E. coli* inactivated quickly during the first 3 days, with densities decreasing to non-detectable levels by day 2 and 3 in the inoculation and control groups, respectively (Fig. 4g). GI in each treatment was about 40% at the initiation of composting and increased gradually as the composting proceeded (Fig. 4h). However, the GI values of the inoculation group were significantly higher ($P < 0.05$) than those of the control group from day 9 to 24. At the completion of the composting period, the GI in both groups reached the maturity requirement of exceeding 80%, with the indexes of 92.2% and 84.4% in inoculation and control groups, respectively.

Discussion

Experiment 1: isolation of microbes from compost

When the internal temperature of the compost decreased to 40 °C (Fig. 2a), the process had nearly finished (de Bertoldi et al. 1983). The temperature of compost decreased to 39.6 °C on day 20, but it then suddenly rose again to 42.3 °C the following day (day 21). Isolation of microorganisms from the overall composting process was terminated on day 22. The reduction in moisture (Fig. 2b) occurred throughout the entire composting process. Similar results were reported by Li et al. (2012) during composting studies using pig manure with bentonite. The pH values changed accordingly with the observed temperature variations (Fig. 2c). Huang et al. (2004) presented similar trends in pH when examining compost containing pig manure and sawdust. These similarities were also found in other studies involving pig manure (Jiang et al. 2014, 2015). The decrease in C/N (Fig. 2d) during composting indicated the biological degradation of organic matter and the availability of nitrogen (Bernal et al. 1998). Values and trends of temperature, moisture, pH and C/N showed the typical features of animal manure composting and that the disposed cattle manure could be considered as non-hazardous according to the Chinese standard of GB7959-2012 (2012).

Xi et al. (2005) revealed that there might be competition between indigenous and inoculated microorganisms in composting, which limited inoculation efficiencies without large amounts of multiplication of inoculation strains. To simulate the natural composting of the manure, the inoculated strains used in this study were isolated from the original indigenous microorganisms and were selected according to their prevalence in Experiment 1.

Effect of Experiment 2: inoculation of compost

The results in Fig. 4 show the effect of inoculation microorganisms. Compared to the control treatment, inoculation with microorganisms did not prolong thermophilic stages (Fig. 4a), which was different from results by Zhang et al. (2013), Xi et al. (2012) and Jiang et al. (2015). All of these previous studies inoculated agricultural waste compost with *P. chrysosporium*, *Aspergillus fumigates*, and NTB agents. However, the temperature values of the inoculation group were significantly higher ($P < 0.001$) than the control group, lasting for 16 days, from day 2 to 17. This is thought to be caused by exogenous and indigenous microorganisms disintegrating the organic matter together, generating large amounts of thermoenergy, similar to that reported by Jiang et al. (2015). Due to the faster disintegration in the inoculation group, the temperature decreased to lower values ($P < 0.05$) than those of the control group, indicating a rapid degradation in the inoculation group. At day 28, the temperature of the inoculation compost decreased to 38.6 °C (< 40 °C), representing that fermentation had nearly finished (de Bertoldi et al. 1983). On day 28, the temperature of the control group was still more than 40 °C. Addition of the inoculation treatment had no significant impact toward shortening composting time, similar to results by Jiang et al. (2015).

The moisture of each pile continuously decreased as the compost aged (Fig. 4b). The optimal water content for composting maintained microbial activities and promoted organic matter decomposition. During composting, a large quantity of water may be used by microorganisms, which will also evaporate during the overall process. As water content diminishes, the rate of decomposition decreases (Bernal et al. 2009). The inoculation group was found to have less loss of moisture when compared to the control group, especially on days 6, 9, 13, 15 and 17 ($P < 0.05$).

Differences in moisture content suggests that those microorganisms in the inoculation pile may be more active than indigenous ones in the control pile.

The pH changed rapidly with the increase and decrease of temperatures (Fig. 4c). Zorpas and Loizidou (2008) reported that the formation of low molecular weight fatty acids and CO_2 emissions from the degradation of organic matter may have contributed to the decrease of pH. Herein, while significant differences ($P < 0.05$) were observed at day 20 and 23, the variations of pH between the two groups were similar. Zhou et al. (2015) presented similar pH trends in both control and inoculation groups of dairy manure with rice straw composting.

Initial C/N ratios of 27.5 in both groups (Fig. 4d) is in the adequate C/N ratio range (25–35). It is considered that microorganisms require 30 parts of C per unit of N (Bishop 1983). There was a larger decrease in the C/N amplitude in the inoculation group over the control group, resulting from strong degradation of the total organic carbon. Similar reports of rapidly dropping C/N ratios in inoculated groups were also found by Zhou et al. (2015). We attribute the increase in C/N at the beginning of the composting process to large quantities of ammonia volatilization (Jiang et al. 2015), with the decrease in the later stages caused by the degradation of organic matter and the mineralization of nitrogen (Bernal et al. 2009; Jiang et al. 2015). In the final compost, both C/N values were less than 12, indicating a satisfactory maturation (Bernal et al. 1998).

During the whole composting process, the organic matter (OM) decreased with time in the two groups due to decomposition by microorganisms (Fig. 4e). Degradation of the OM reduces the weight of the pile and decreases gradually as composting progresses due to the reduction in available carbon sources. Eventually, synthesis reactions of new complexs and polymerized organic compounds (humification) prevail over mineralization during the maturation phase (Bernal et al. 2009).

The number of microorganisms (Fig. 4f) observed in the inoculation group was higher than that in the control group (expected), which likely contributed to the faster degradation of organic matter seen in Fig. 4e. The number of microorganisms in the inoculation group increased rapidly ($P < 0.05$) during the first 6 days over that of the control group, corresponding directly to the increase in temperature observed in Fig. 4a. The inoculated bacteria were not expected to contribute significantly to the whole composting process, but they may have contributed in the early phases for the inoculation group, as evidenced by the rapid growth of microorganisms. The decrease of *E. coli* in cell density is suggested to be related to the rapid increase of temperature (Fig. 4g), as pathogenic microorganisms are killed with temperatures above 55 °C (Miller 1992).

GI, an important maturity indicator, was used to determine the phytotoxicity for crops after organic amendments in agriculture (Tiquia and Tam 1998). In this study, the GI in both groups reached the maturity requirement of exceeding 80%, with the indexes of 92.2% and 84.4% in the inoculation and control groups, respectively (Fig. 4h). Wang et al. (2011) found that inoculating with *Penicillium expansum* could cause a higher GI in the final compost. Similar result was also observed by Jiang et al. (2015) with the addition of 1% NTB agent at the beginning of the composting period. Inoculated microorganisms may have quickly remove phytotoxic substances such as NH_3 and organic acids (Wang et al. 2011), leading to a higher GI index.

Conclusions

The inoculation mixture containing 12 microorganisms, including 2 strains of *Bacillus licheniformis*, 3 strains of *Bacillus megaterium*, and single strains of *Bacillus thuringiensis*, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Cellulosimicrobium funkei*, *Cellulomonas* sp., *Thermomonospora* sp. and *Streptomyces* sp., did not significantly shorten composting time. However, the pile temperature was increased, the degradation of organic matter was accelerated, and significant higher GI index indicated the maturity was promoted by inoculation microorganism. Results suggest that the finished compost would provide value as alternative source of nutrients for plants. Further studies are needed to investigate the mechanisms employed by the selected microorganism strains used in this study.

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Author contributions JL, XW, CC and LW did the experiments. FH and YW helped in the data analysis. YX and XL gave many constructive suggestions. LW, the corresponding author, designed this study.

Compliance with ethical standards

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

Ethical statements This article does not contain any studies with human participants or animals performed by any of the authors.

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