Effects of High Temperature Pretreatment and Inoculation of *Bacillus coagulans* on Promoting Aerobic Composting of Chicken Manure

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Effects of high temperature pretreatment and inoculation of *Bacillus coagulans* were determined relative to the physicochemical properties and bacterial community of aerobic composting of chicken manure. Chicken manure was pretreated with high temperature for 0 h (CJ), 0.5 h (T-0.5h), 1.0 h (T-1.0h), 1.5 h (T-1.5h), and 2.0 h (T-2.0h) and then inoculated with *B. coagulans*. Chicken manure without high temperature pretreatment was included as control (CK). The results showed that the temperature of manure in CJ, T-0.5h, T-1.0h, T-1.5h, and T-2.0h groups was 2.2 to 8.4 °C higher than the chicken manure in CK within 1 day. On day 3, the chicken manure temperature reached a peak, which was 1.5 to 7.7 °C higher than that in the CK (56.8 °C). Both inoculation of *B. coagulans* and high temperature pretreatment increased the abundance and diversity of the bacterial community. The abundance of *Firmicutes* in T-1.5h was significantly higher than that in CJ. In the temperature decreasing period, the abundance of *Bacillus* in T-1.5h group was significantly higher than that in the CK and CJ. Overall, it was concluded that high temperature pretreatment and *B. coagulans* inoculation can accelerate the temperature elevation, increase the temperature of compost, and regulate the structure of bacterial community.

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INTRODUCTION

With the industrialization, large-scale and intensive development of China's livestock and poultry breeding industry, the annual production of livestock and poultry manure has posed a huge challenge to ecological environment management. In recent years, the annual output of livestock and poultry manure in China has reached 3.8 billion tons (Yao *et al.* 2021). However, the comprehensive resource utilization rate is less than 60%. Aerobic composting is an effective way to remove and eliminate organic wastes such as livestock and poultry manure. This process utilizes microorganisms under aerobic conditions to transform organic wastes into fertilizers that are beneficial to soil improvement and crop growth, thereby achieving harmless reduction and recycling of the organic waste (Chen *et al.* 2022; Fu *et al.* 2022).

Traditional aerobic composting has the disadvantage of long composting period and low efficiency, which restricts the development of the composting industry (Zhang *et al.* 2021b). Previous studies have explored various approaches including optimization of the composting conditions and raw material pretreatment to achieve rapid high-temperature composting, shortening the composting period, and improving the humification efficiency. High temperature pretreatment has been found to promote rapid decomposing of livestock and poultry manure and shortens the composting period (Huang *et al.* 2019). Huang *et al.* (2019) showed that high temperature pretreatment extends the high temperature period of composting, enhances the synthesis of humic substances precursors, promotes the formation of humic substances, and accelerates the process of composting. In addition, microorganisms are the internal driving force of biotransformation (Wang *et al.* 2022); the inoculation of exogenous microorganisms in aerobic compost has been found to improve the compost maturity and quality (Hu *et al.* 2021). Li *et al.* (2020) found that microbial inoculants do not shorten the composting time of cow manure, but they increase the compost temperature, accelerate the degradation of organic matter, and promote its humification efficiency. Zhang *et al.* (2021a) inoculated rice straw compost with *Bacillus*, and the results showed that *Bacillus* inoculation indirectly affects the degradation of lignocellulose by changing the microbial community structure in addition to its direct degradation effect.

To optimize the composting process, Cao *et al.* (2017) inoculated fresh chicken manure with microorganisms after high temperature pretreatment, and determined the effect of high temperature pretreatment on chicken manure composting by analyzing the physical and chemical properties and nutrients. However, the effect of inoculating microorganisms on chicken manure after high temperature pretreatment has not been studied systematically in terms of microbial community changes. Therefore, this study analyzed the effect of *B. coagulans* inoculation after high-temperature pretreatment on physicochemical properties and bacterial communities of chicken manure compost.

EXPERIMENTAL

Experimental Materials

The chicken manure was obtained from a farm in Jinan City, Shandong Province, and air-dried before use. The basic properties of the manure (shown in Table 1) are based on a test of dried chicken manure. The *B. coagulans* agent was composed of *B. coagulans* and vermiculite, in the shape of a solid powder, and the inoculum size was 5.0×10^9 CFU/g. The inoculation method was to turn the material to make it evenly mixed with the material when spraying the *B. coagulans*.

Table 1. Basic Properties of the Manure

Experimental materials	Water content (%)	Total organic carbon (%)	Total nitrogen (%)	Total phosphorus (%)	Total potassium (%)	Carbon/ nitrogen ratio
Chicken manure	3.02±0.03	22.85±0.12	1.98±0.01	3.18±0.01	3.69±0.02	11.54

Experimental Design

Chicken manure (3 kg) was mixed with water, and the water content was adjusted to approximately 60%. Chicken manure was pretreated for 0.5, 1.0, 1.5, and 2.0 h in the

dry incubator, which was adjusted to 80 °C. The corresponding conditions were designated as T-0.5h, T-1.0h, T-1.5h, and T-2.0h groups, respectively. After cooling to room temperature, manure was inoculated with 0.1% (w/w) *B. coagulans* bactericide. Chicken manure without treatment was included as control (CK). In addition, pure chicken manure supplemented with 0.01%(w/w) *B. coagulans* was the bacterial-additive group CJ. The composting was carried out in the foam box (54*39*29cm), and the height of the compost material was about 20 cm. Three replicates were included for each group. The total experimental period was 6 days. Manure in all treatment groups was turned over once a day. On days 0, 3, and 6, 200-g samples were collected from the top, middle, and bottom of each compost and mixed evenly to determine the moisture content, pH and bacterial community structure.

Methods for the Determination of Each Parameter

Temperature was measured directly with a thermometer, which was inserted 10 cm in the center of the pile at 10:00 and 14:00 every day. Water content was measured by drying method at 105 °C. Air-dried samples were passed through a 1 mm sieve, and water was added at a solid-liquid ratio of 1:10 (w/ v) for extraction. Supernatant was tested with a pH meter (Ajmal *et al.* 2021).

Methods for Bacterial Community Analysis

The samples of CK, CJ, and T-1.5h groups were collected at the initial stage (day 0), high temperature period (day 3), and cooling period (day 6). Samples were labelled as CK1, CK2, and CK3 (for CK samples collected on day 0, day 3, and day 6, respectively), CJ1, CJ2 and CJ3 (for CJ samples collected on day 0, day 3 and day 6, respectively), and H_1, H_2 and H_3 (for T-1.5h samples collected on day 0, day 3 and day 6, respectively). High-throughput sequencing and analysis were performed by Illumina MiSeq after PE library construction (Wuhan Baiyi Huineng Biotechnology Co., Ltd.).

DNA extraction

The sample was taken out of the refrigerator, and as soon as possible the appropriate amount of sample (between 0.2 and 0.5 g) was added to the centrifuge tube containing the extract lysate for grinding. For pretreated samples, nucleic acid was extracted by OMEGA Soil DNA Kit (D5635-02) (Omega Bio-Tek, Norcross, GA, USA) kit. The extracted DNA was determined by 0.8% agarose gel electrophoresis, and the DNA was quantified by Nanodrop.

16SrRNA gene amplification

The bacteria project selected the highly variable V3V4 region of the bacterial 16S rRNA gene with a length of about 468bp for sequencing. Bacterial 16S rRNA V3-V4 region specific primers were selected for polymerase chain reaction (PCR) amplification, 338F(5'-barcode+ACTCCTACGGGAGGCAGCA-3'), 806R(5'-GGACTACHVGGGTW-TCTAAT-3'). The barcode in the front primer was a 7-10 base oligonucleotide sequence used to distinguish between different samples in the same library.

Conditions of DNA amplification

After the components required for the PCR reaction were configured, the template DNA was fully denatured on the PCR apparatus at 98 ℃ for 5 min, and then the amplification cycle was entered. In each cycle, the template was denatured by holding for

30 minutes below 98 ℃, and then the temperature was lowered to 53 ℃ for 30 seconds to fully anneal the primer and template. It was held at 72 ℃ for 45 s, so that the primer extends on the template and synthesizes DNA, completing a cycle. This cycle was repeated 25 times, enriching the amplified DNA. Finally, the product was kept at 72 ℃ for 5min, so that the extension of the product was complete, and it was stored at 12 ℃. The results were amplified by 2% agarose gel electrophoresis. The target fragments were cut and recovered with an Axygen gel recovery kit.

Data Analysis

The data was analyzed by Origin 2021 software, and the microbial sequencing data were provided by Wuhan Baiyi Huineng Biotechnology Co., Ltd.

RESULTS AND DISCUSSION

Variation of Temperature and Water Content during Composting

Temperature is the most critical factor affecting composting (Chen *et al.* 2020; Cui *et al.* 2021). It is easier to degrade the refractory substances in the composting process under higher temperature condition (Hosseini and Abdul Aziz 2013). Dynamic changes of the composting temperature in this study were as shown in Fig. 1A. The whole composting process experienced three stages: temperature elevating (heating) period, high temperature period, and temperature decreasing (cooling) period, which is similar to what has been observed in previous studies (Qiu *et al.* 2019). The compost temperature in the T-0.5h, T-1.0h, T-1.5h, and T-2.0h groups increased rapidly and within 1 day, and it reached 51.3 ℃, 51.3 ℃, 53.5 ℃, and 51.0 ℃, respectively, which was 5.9 to 8.4 ℃ higher than that in CK (45.1℃). On day 3, the temperature reached peak (61.8 ℃, 61.5 ℃, 64.5 ℃, and 63.8 ℃ in T-0.5h, T-1.0h, T-1.5h, and T-2.0h groups, respectively), which was 4.7 to 7.7 ℃ higher than that in CK (56.8 ℃). The temperature of compost in CJ group was 47.3 °C on day 1 and 58.3 °C on day 3, which was only 2.2 °C and 1.5 °C higher than that in CK group.

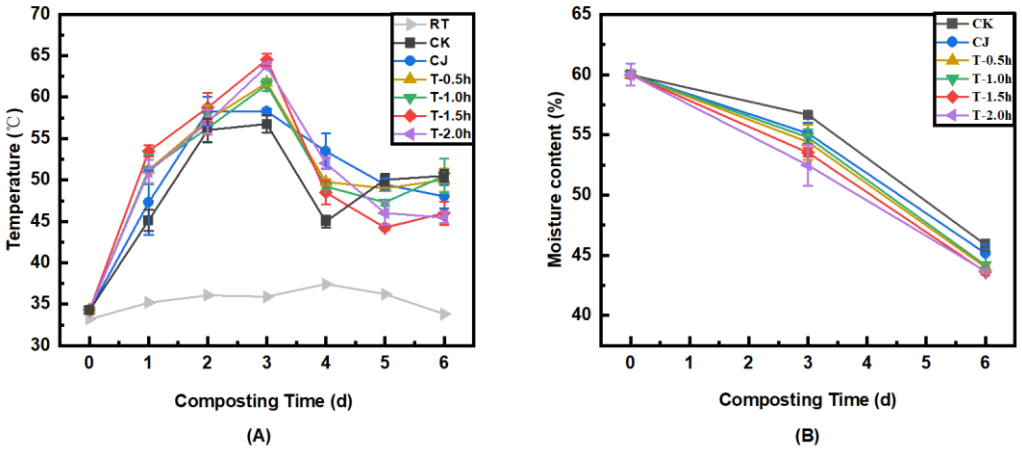

Fig. 1. Changes in temperature (A) and water content (B) during composting

Water is important for maintaining microorganisms in compost (Li *et al.* 2021). With increased compost temperature, the water content of the manure pile gradually decreased (Fig. 1B). On day 3, the temperature increasing rate and temperature peak of the compost in CJ, T-0.5h, T-1.0h, T-1.5h, and T-2.0h groups were higher than those in the

CK. The water content in CJ, T-0.5h, T-1.0h, T-1.5h, and T-2.0h groups decreased by 1.50%, 2.26%, 1.83%, 3.09%, and 4.17% compared with that in the CK group (56.64%). On day 6, the water content was also decreased by 0.68 to 2.22% compared with CK. These results indicate that high temperature pretreatment increased the temperature elevation speed and temperature peak of compost, reduced the water content, and promoted the composting process.

Variation of the pH Value during Composting

The pH value of the compost can affect the life activities of microorganisms, thereby affecting the degradation efficiency of organic matter and reflecting the composting process (Liu *et al.* 2021). The pH of the manure stack changed with time (Table. 2). The pH value of the compost raw material after high temperature pretreatment was 0.02 to 0.28 units higher than that of CK (pH=8.51). In the early stage of composting, due to the low C/N ratio in chicken manure, NH_3 was generated during the decomposition of nitrogen-containing substances, which led to an increase of pH (Yu *et al.* 2019). On day 3, pH value in T-0.5h, T-1.0h, T-1.5h, and T-2.0h reached 9.00, 9.15, 9.18, and 9.26, respectively, which was 0.04 to 0.30 higher than that in CK (pH=8.96). This result indicated that high temperature pretreatment accelerated the degradation of the substrate and the composting process.

Table 2. Variation of pH During Composting

	CK	CJ	T-0.5h	T-1.0h	T-1.5h	T-2.0h
0d	8.60±0.13	8.53±0.03	8.56±0.00	8.76±0.03	8.77±0.01	8.81±0.05
3d	8.97±0.01	8.57±0.03	9.00±0.04	9.16±0.07	9.18±0.02	9.26±0.04
6d	8.89±0.02	8.73±0.08	9.08±0.01	9.14±0.04	9.20±0.05	9.19±0.04

Effect of High Temperature Pretreatment and *B. coagulans* Inoculation on the Bacterial Community of the Compost

The basic goal of composting is that the organic matter is rapidly degraded into stable humus under the microbial life activities (Shangguan *et al.* 2020). In this study, the temperature peak of T-1.5h group reached 64.5 °C, which was 2.75 °C and 3 °C higher than that of T-0.5h and T-1.0h, respectively, and the temperature elevation was also relatively fast. Therefore, T-1.5h, CJ, and CK were selected for bacterial community analysis during the process of composting.

Microbial diversity plays an important role in maintaining microbial ecology and is an important indicator of microbial species richness and community structure (Du *et al.* 2019). The Alpha diversity index of bacterial communities of the samples is shown in Table 3 and Fig. 2. The Coverage index represents the coverage of the sample library and higher Coverage index would indicate that the sequencing results are closer to the real situation of the sample. The Coverage index of each sample in this study reached over 99%, indicating that the amount of sequencing data was sufficient to represent the real situation of the bacterial community in the sample. Chao1 index and Shannon index represent the abundance and diversity of the bacterial community of the sample. The present results showed that the Chao1 index and Shannon index in CK, CJ, and T-1.5h groups increased initially and then decreased (Fig. 2A). Compared with CK, both the abundance and the diversity in CJ were significantly increased at the initial stage of composting, suggesting that inoculation with *B. coagulans* increased the abundance and diversity of bacterial

communities. The abundance of bacterial community in the T-1.5h group was lower than that in CJ group, which could be due to the increase of temperature and the inactivation of some bacteria. However, the diversity of bacterial community in T-1.5 was 22.79% higher than that in CJ, indicating that high temperature pretreatment increased compost microbial diversity. Figure 2B showed the Beta diversity of microorganisms of the sample, representing the similarity index between each sample (Liu *et al.* 2020). With the progress of composting, the bacterial communities in CK, CJ, and T-1.5h groups all changed significantly. The bacterial community composition of each group was similar at the initial stage of composting. However, the bacterial community composition in the high (peak) temperature period and the temperature decreasing (cooling) period were significantly different among CK, CJ, and T-1.5h groups. In addition, the abundance and diversity of the bacterial community also changed significantly, indicating that high temperature pretreatment and inoculation of *B. coagulans* can change the richness and diversity of bacterial community, and adjust bacterial community structure.

Table 3. Coverage Index of Bacterial Community

Sample	CK1	CK2	CK3	CJ1	CJ2	CJ3	H_1	H_2	H_3
Coverage	0.99742	0.99569	0.99456	0.99284	0.99091	0.99116	0.99593	0.99594	0.99731

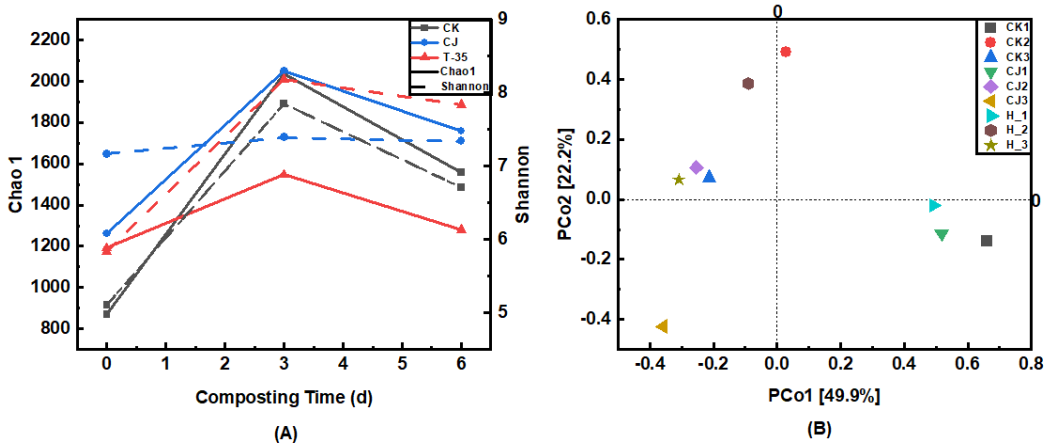

Fig. 2. (A) Alpha diversity index and (B) beta diversity of compost bacterial community

Effect of High Temperature Pretreatment and *B. coagulans* Inoculation on Microorganism Composition during Composting

Figure 3A shows the number of taxa in each sample. The number of taxa in H_1 was significantly higher than that in CJ1 and CK1. In temperature decreasing period, the number of taxa in T-1.5h was also significantly different from that in CK and CJ, indicating that high temperature pretreatment changed the bacterial community structure. Figure 3B shows the main phylum of bacteria in the samples during the composting process. *Firmicutes, Proteobacteria, Actinomycetes* and *Bacteroidetes* were the dominant genera in the process of chicken manure composting, accounting for 94.3% to 99.8% of the total bacteria, which is consistent previous studies (Zhong *et al.* 2018; Ma *et al.* 2022). *Firmicutes* and *Proteobacteria* were dominant in CK, CJ and T-1.5h at the initial stage of composting and changed significantly during the composting process.

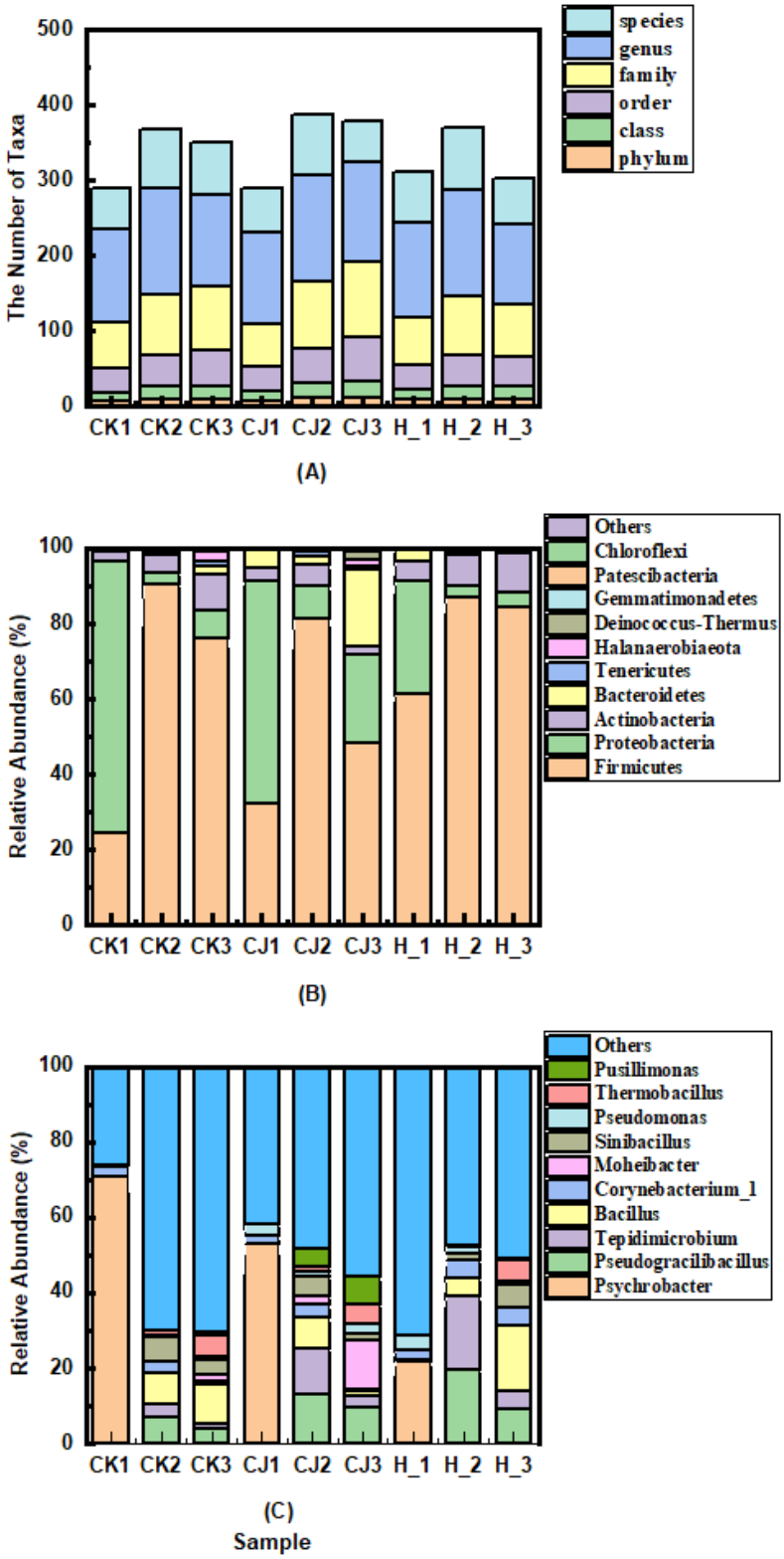

Fig. 3. Bacterial community structure (A) Number of taxa (B) Phylum level (C) Genus level

After high temperature pretreatment, the abundance of *Firmicutes* increased in T-1.5h, which was 36.6% and 28.7% higher than in the CK and CJ groups. It was speculated that high temperature pretreatment promoted the colonization of *B. coagulans* (*B. coagulans* belongs to the phylum *Firmicutes*) or inactivated some other bacteria, thereby increasing the abundance of *Firmicutes. Firmicutes* in CJ only dominated in the high temperature period, and the dominance decreased in the temperature decreasing period. Unlike CJ, *Firmicutes* in T-1.5h also dominated in the temperature decreasing period (84.3%), which was 35.8% higher than that in CJ (48.4%). Studies have shown that the predominance of *Firmicutes* in the high temperature period could be due to the thick cell wall and the thermotolerance of endospores (Galperin 2013). The abundance of *Proteobacteria* changed in the opposite direction to that of *Firmicutes*.

The presence of highly abundant *Actinobacteria* in the composting process is one of the signs of composting maturity (Wang *et al.* 2014). With the progress of composting, *Actinobacteria* in CJ did not change significantly, while the abundance of *Actinobacteria* in T-1.5h gradually increased, reaching 10.61% on day 6 of composting. This result was 4.18 times higher than that in CJ (2.54%), indicating that high temperature pretreatment promotes decomposing of the compost.

At the bacterial genus level, the abundance of *Psychrobacter* in T-1.5h decreased significantly by 49.9% and 30.9% compared with that in CK (70.8%) and CJ (52.8%), respectively. In the high temperature period, *Psychrobacter* could not be detected in CK, CJ and T-1.5h, indicating that high temperature led to the inactivation of *Psychrobacter*. At this time, *Pseudogracilibacillus* and *Tepidimicrobium* dominated, and the abundance of both in T-1.5h was 7% higher than that in CJ. However, in the temperature decreasing period, their abundance was not significantly different between T-1.5h and CJ. The genus *Pseudomonas* in *Proteobacteria* has high ammonification activity and denitrification activities (Cui *et al.* 2019), which contributes to the rapid temperature increases of composting (Ma *et al.* 2022). In the initial stage of composting, the abundance of *Pseudomonas* in T-1.5h was increased by 2.61% and 0.89%, respectively, compared with that in CK (1.46%) and CJ (3.18%) (Fig. 3C), which promoted the temperature elevation of the compost (Fig. 1A). *B. coagulans* belongs to the genus *Bacillus*. The abundance of *Bacillus* in CJ was not significantly different from that in CK in the temperature elevation and peak temperature periods. In the temperature decreasing period, the abundance of *Bacillus* in CJ was significantly decreased. In the high temperature period, the abundance of *Bacillus* in T-1.5h was lower than that in CK and CJ. However, in the temperature decreasing period, *Bacillus* dominated in the T-1.5h group (17.6%), which was 7.2% and 16.2% higher than that in the CK (10.4%) and CJ (1.35%) groups, indicating that the high temperature pretreatment improved the colonization of *Bacillus*.

No matter from the phylum level or the genus level, the bacterial community composition of CK, CJ and T-1.5h groups differed significantly at different composting stages, indicating that the bacterial community had significant renewal or replacement during the composting process. The bacterial community composition at the same composting stage was also significantly different between different groups, and the abundance of *Firmicutes* and *Bacillus* was significantly increased after high temperature pretreatment, while the abundance of *Psychrobacter* decreased, indicating that high temperature pretreatment can change the bacterial community structure, accelerate the temperature elevation of the compost, and may improve the colonization of *B. coagulans*.

Pearson Correlation Analysis between Bacterial Community and Physicochemical Properties

The Pearson correlation analyses of the dominant bacterial phyla in the CK, CJ, and T-1.5h groups with temperature, water content, and pH are shown Fig. 5. The results showed that after inoculation with *B. coagulans* the overall correlation changed, in which the correlation coefficient increases accounted for 28.6%, the correlation coefficient decreases accounted for 42.9%, and the correlation coefficient was changed by 28.6% (Fig. 4B), indicating that *B. coagulans* altered the correlation of temperature, water content and pH with bacterial communities.

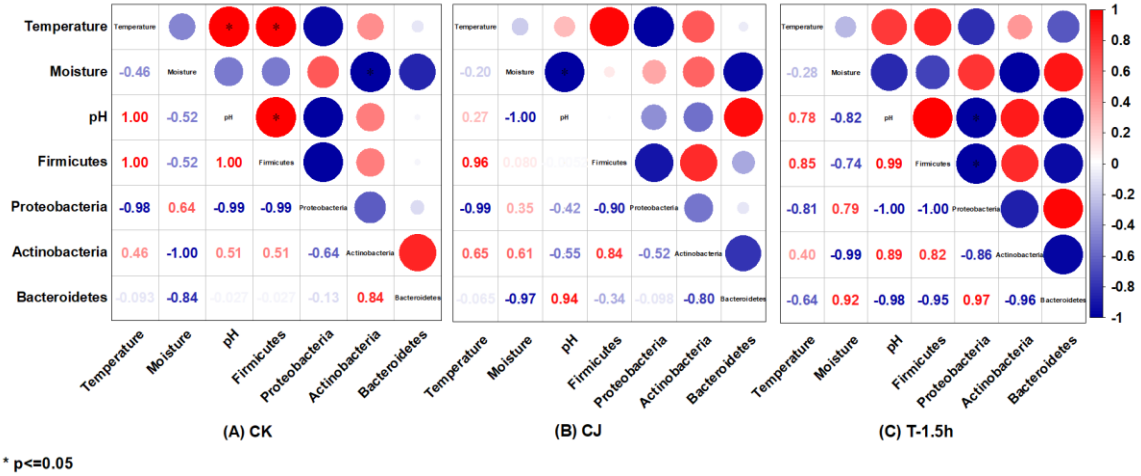

Fig. 4. Pearson correlation analysis of microbial communities with physicochemical properties in CK (A), CJ (B) and T-1.5h (C)

After high temperature pretreatment, the correlation coefficient increases accounted for 42.9%, the correlation coefficient decreases accounted for 23.8%, and the correlation coefficient was changed by 33.3% compared with that in CJ group (Fig. 4C). The positive correlation of *Firmicutes* with temperature was decreased by 11%, and the negative correlation with *Bacteroidetes* was increased by 61%. The correlation with water content and pH changed. The negative correlation between *Proteobacteria* and temperature decreased. After high temperature treatment, the abundance of *Proteobacteria* increased compared with CJ group, which increased the temperature elevation of the compost. Pearson correlation analysis showed that high temperature pretreatment could change the correlation of bacterial community with temperature, water content and pH of compost, which accelerates the temperature elevation and increases the compost temperature.

Based on the diversity and Pearson correlation analysis, it can be concluded that the abundance and diversity of bacterial community increased significantly after inoculation with *B. coagulans*. After high temperature pretreatment, the abundance of *Firmicutes* was increased compared with CJ in the temperature elevating, high temperature and temperature decreasing periods. The correlation between bacterial community and physical and chemical indicators also changed, and bacterial community changed significantly, which increased the temperature elevation speed and the temperature of the compost and may improve the colonization of *B. coagulans*.

CONCLUSIONS

1. After the chicken manure was pretreated with high temperature (80 °C), the temperature elevation speed and the temperature peak of the compost were significantly increased, which accelerated the declining of the water content in the compost. Pretreatment for 1.5 h results in the fastest temperature elevation and highest temperature peak, which accelerated the composting process.
2. After inoculation with *B. coagulans*, the abundance and diversity of the compost bacterial community increased significantly, and the community structure also changed significantly. After high temperature pretreatment, the abundance of *Firmicutes* and *Bacillus* increased significantly, indicating that high temperature pretreatment may promote the colonization of *B. coagulans* and adjust the structure of bacterial community. The correlation of bacterial community with temperature, water content, and pH also changed accordingly. After high temperature pretreatment, the negative correlation between *Proteobacteria* and temperature decreased. The abundance decreased compared with CK and CJ, which accelerated the temperature elevation, indicating that high temperature pretreatment can promote temperature elevation during the process of composting.

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