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# Effect of Thermophilic Microbial Agents on Crude Fiber Content, Carbohydrate-Active Enzyme Genes, and Microbial Communities during Chinese Medicine Residue Composting

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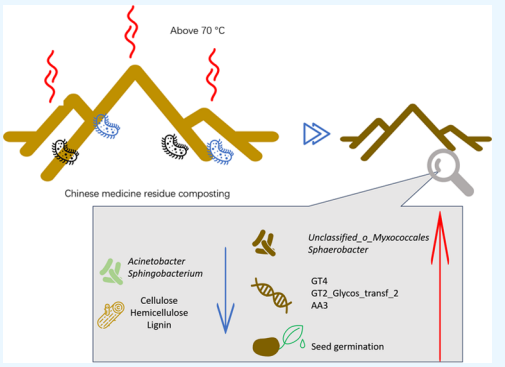
**ABSTRACT:** The objective of this study was to investigate the impact of thermophilic bacteria on crude fiber content, carbohydrate-active enzyme (CAZyme) genes, and associated microbial communities during Chinese medicine residues composting. The study examines changes over 15 days of composting with (*T*) and without (CK) thermophilic microbial agents. Results show that the group *T* compost temperature reached a maximum of 71.0 °C and remained above 70 °C for 2 days, while the group CK maximum temperature was only 60.9 °C. On Day 15, the seed germination index (GI) of group *T* reached 98.7%, while the group CK GI was only 56.7%. After composting, the degradation rates of cellulose, hemicellulose, and lignin in group *T* increased by 5.1, 22.5, and 18.5%, respectively, compared to those in group CK. Thermophilic microbial agents changed the microbial communities related to CAZymes, increasing *unclassified_o_Myxococcales* and *Sphaerobacter* abundance and reducing *Acinetobacter* and *Sphingobacterium* abundance. Thermophilic microbial agents also increased the abundance of the GT4, GT2_Glycos_transf_2, and AA3 gene families. These results show that thermophilic microbial agents can increase composting temperature, accelerate compost maturation, and promote crude fiber degradation. Therefore, they have broad application potential.




## 1. INTRODUCTION

Traditional Chinese medicine plays an important role in medical treatment in China. Traditional Chinese medicine can generally be divided into three categories: plant-based medicine, animal-based medicine, and mineral-based medicine. Among them, plant-based Chinese medicines account for 87% of all Chinese medicines.[1] Chinese medicine residue is the waste generated after the effective components of traditional Chinese medicine are extracted from plants. Due to the continuous development of the pharmaceutical industry in China, discharges of Chinese medicine residues (CMR) have also been increasing. According to statistics, China produces 70 million tons of CMR every year, and this trend is increasing yearly.[2] CMR can be treated by composting. CMR contain carbohydrates, proteins, crude fiber, polysaccharides, nitrogen, phosphorus, potassium, and other nutrients, which are suitable for use as a fertilizer.[3] Compared with other organic fertilizers, those made from traditional CMR are better at increasing soil nutrient levels, promoting soil polymerization, improving crop yield, and reducing heavy metal pollution, so they represent a source of high-quality organic fertilizer.[4]

CMR is rich in crude fiber, which is difficult to degrade during composting and impedes compost maturation.[5] Many studies have focused on composting materials that are rich in crude fiber, such as cow dung and straw. However, there are a few reports on the degradation of crude fiber from CMR during thermophilic composting. Awasthi and Li et al. found that the addition of microbial agents to cow manure compost can effectively promote the degradation of cellulose.[6,7] Wang et al. found that actinomycetes affect the degradation of crude fiber in straw compost.[8] CMR is similar to cow dung and straw, which are also rich in crude fiber. Therefore, it might be feasible to promote the degradation of crude fiber in CMR by the addition of microbial agents.

Carbohydrate-active enzymes (CAZymes) play an important role in the metabolism of glycosidic bonds. An in-depth study of CAZymes is of great significance for understanding the metabolism of crude fiber. The CAZy database (http://www.cazy.org/) is a scientific database for enzymes that synthesize or decompose complex carbohydrates and glycohydrates,[9] which can be used to analyze changes in enzyme activity during the metabolism of crude fiber. At present, an increasing

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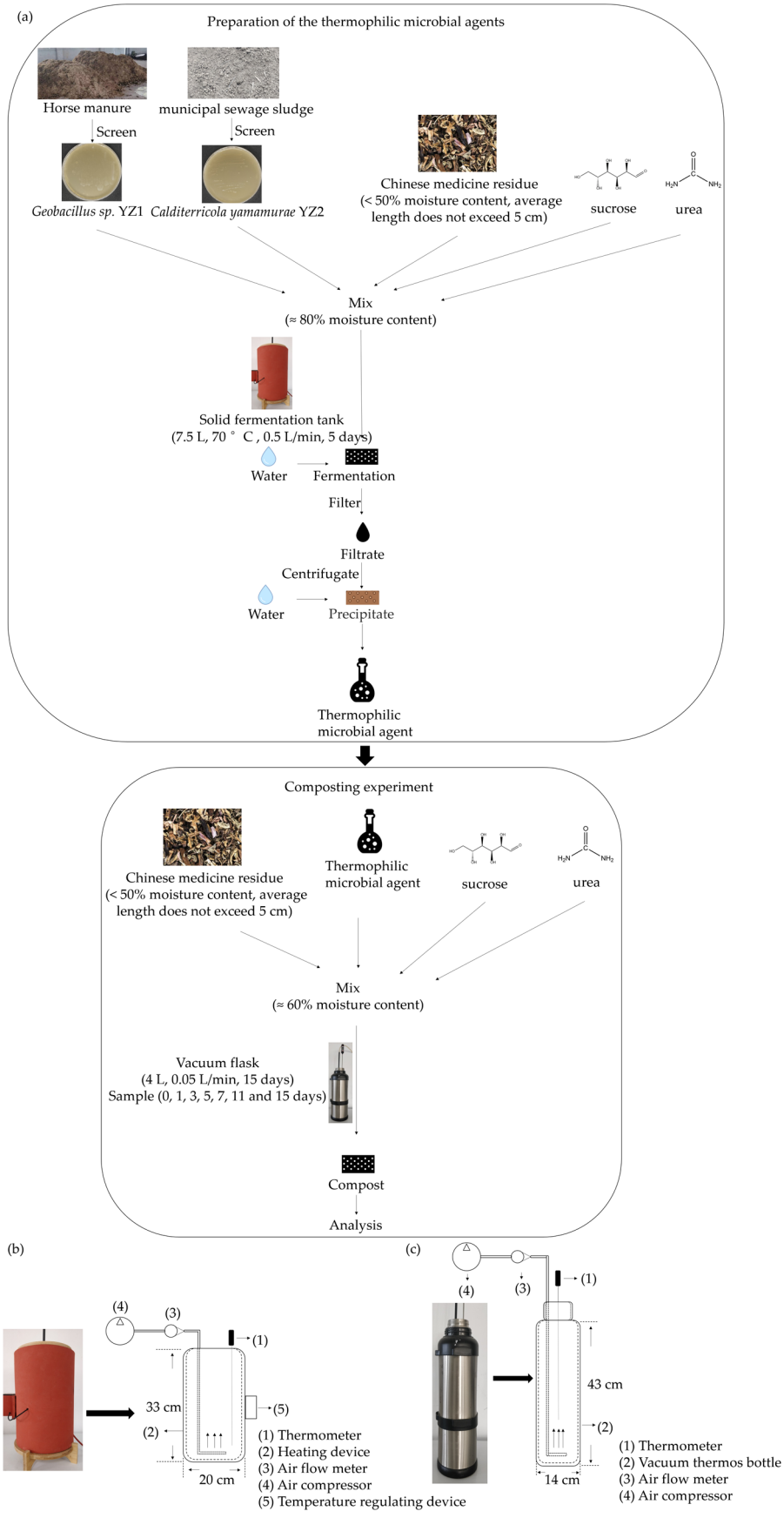


**Figure 1.** (a) Workflow chart; (b) fermentation system; (c) bench-scale composting system.



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number of studies on crude fiber have been added to the database for analysis. For example, Ayme et al. screened enzymes with lignocellulosic decomposition activity by using metagenomic and CAZy databases to characterize compost.[10] Understanding the changes that occur in crude fiber during CMR composting by studying the abundance of CAZyme-related genes is of practical significance.

Temperature is considered one of the most important factors affecting composting efficiency.[11] High temperature plays an important role in accelerating compost maturation and materials transformation.[12] Chang et al. increased the composting temperature of food factory wastes; as a result, composting time was decreased to 4 days.[13] Elango et al. used thermophilic composting to treat municipal solid wastes, which reduced composting time to less than 40 days.[14] Song et al. used thermophilic composting technology to shorten the maturation time of sheep manure organic fertilizer by 50%, and the compost reached maturity within 27 days.[15] In addition, Gu et al. found that high temperatures can promote the degradation and mineralization of cellulose.[16] However, due to a lack of thermophilic bacteria in the CMR, composting efficiency is often lower than expected. To date, most researchers have increased composting temperature through the inoculation of thermophilic bacteria to accelerate the biodegradation of organic matter, shorten composting cycles, and improve composting efficiency.[17,18] For example, Xue et al. successfully used YM thermophilic bacteria for sludge thermophilic composting.[19] The liquid thermophilic bacteria developed by Liao's team have also been successfully applied for the thermophilic composting of sludge and cow dung.[20]

At present, few reports exist on the application of thermophilic bacteria in CMR composting, especially with respect to the effects of thermophilic bacteria on the changes in crude fiber, CAZyme-related functional genes, and microbial communities during composting. Therefore, in this study, thermophilic bacteria were obtained by enrichment and added to CMR for evaluation. The main objectives were (1) to explore the effects of thermophilic bacteria on the basic physical and chemical indexes of CMR during composting; (2) to explore the effect of thermophilic bacteria on the abundance of CAZyme-related functional genes and microbial communities; and (3) to explore correlations among environmental factors, functional genes, and microbial communities. Through this study, we can better understand the effect of thermophilic bacteria on the degradation of crude fiber during CMR composting, which is of great significance in promoting the degradation of crude fiber and expanding the application of thermophilic bacteria.

## 2. MATERIALS AND METHODS

**2.1. Preparation of Thermophilic Microbial Agents.** The procedures used in this study are listed in Figure 1a. The sources of the raw materials used in this study are listed in Table 1. In this study, the components of the CMR were Taraxacum mongolicum, *Scutellaria baicalensis*, Corydalis bungeana, and Strobilanthes cusia. During the preliminary screening process, we found the strains *Geobacillus* sp. YZ1 and *Calditerricola yamamurae* YZ2 were able to grow using the Chinese medicinal residues as substrates at 70 °C. Therefore, they were selected as thermophilic microbial agents for composting of the CMRs. In addition, *Geobacillus* sp. YZ1 and *C. yamamurae* YZ2 were obtained from horse manure and municipal sewage sludge, respectively. They were added to the CMR for fermentation to enrich the CMR in thermophilic bacteria.

**Table 1. Source of Samples**

| name | sampling site |
|---|---|
| Chinese medicine residue | Jiangsu Chinese Medicine Factory |
| horse manure | Shanghai Wild Animal Park |
| municipal sewage sludge | Shanghai Fengxian Sewage Sludge Treatment Plant |

First, we used a solid/liquid separator to dehydrate the fresh CMR to approximately 50%. Then, we cut the CMR with a knife so that the average length did not exceed 5 cm. The CMR was not sterilized and was used as the raw material. The water content of the CMR was adjusted to 80% with sterile water, and then 1 kg of CMR containing a water content of approximately 80% was mixed with 0.5 g of sucrose, 0.5 g of urea, 1 mL of approximately 1 × 10 10 CFU strain YZ1 and 1 mL of approximately 1 × 10 10 CFU strain YZ2. After uniform mixing, the mixture was placed in a solid 7.5 L fermentation tank (Figure 1b) at 70 °C for 5 days, and the ventilation rate was maintained at 0.5 L/min for the enrichment of thermophilic bacteria. During fermentation, sterile water was added to keep the water content of the CMR above 60%. We added 1 L of sterile water to 100 g of fermented CMR (dry weight), agitated the suspension for 10 min, filtered it with filter paper, and collected the filtrate. The filtrate was centrifuged at 7000 rpm for 8 min. We retained the precipitate and resuspended it in 100 mL of sterile water. The liquid obtained contained the thermophilic microbial agents used for CMR composting.

**2.2. Composting Experiment.** A total of 250 mL of inoculum, 1.25 g of sucrose, and 1.25 g of urea were added to the CMR with 50% water content per kg as the experimental group (group *T*), and 250 mL of sterile water, 1.25 g of sucrose, and 1.25 g of urea were added to the CMR in the control group (group CK). The basic physical and chemical properties of the raw materials are shown in Table 2. As shown in Figure 1c, the composting experiment was carried out in a 4 L vacuum thermos flask for 15 days. Each bottle contained 1 kg of compost material, and the ventilation rate at the bottom of the bottle was 0.05 L/min. All treatments were repeated 3 times. Approximately 50 g of sample was collected from each of the three bottles for each treatment on days 0, 1, 3, 5, 7, 11, and 15. Each collected sample was divided into two parts: one part was stored at 4 °C for the determination of

**Table 2. Physicochemical Properties of the Chinese Medicine Residue**

| index | CK | *T* |
|---|---|---|
| moisture (%) | 61.4 ± 0.8 | 62.5 ± 0.7 |
| pH | 7.2 ± 0.1 | 7.2 ± 0.1 |
| EC (μS/cm) | 488.1 ± 12.5 | 529.0 ± 26.4 |
| TOC (%)[a] | 39.2 ± 0.6 | 38.8 ± 0.5 |
| TN (%)[a] | 2.5 ± 0.01 | 2.5 ± 0.04 |
| C/N[a] | 15.7 ± 0.2 | 15.7 ± 0.4 |
| cellulose (g/kg)[a] | 17.6 ± 1.0 | 17.8 ± 2.1 |
| hemicellulose (g/kg)[a] | 8.4 ± 0.3 | 8.4 ± 0.2 |
| lignin (g/kg)[a] | 55.6 ± 1.5 | 54.7 ± 0.1 |

[a]Calculated on a dry weight basis.



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physicochemical indexes, and the other part was kept at −80 °C for the extraction of DNA.

**2.3. Determination of Physicochemical Indexes.** Temperature, water content, pH, electrical conductivity (EC), seed germination index (GI), total organic carbon (TOC), total nitrogen (TN), and the ratio of TOC to TN (C/N) were determined in accordance with methods reported in our previous study.[21] In addition, the method for cellulose, hemicellulose, and lignin determination was as follows: a filter paper bag was dried, weighed, and denoted m0 (unit: mg). A sample was dried to a constant weight, 1000 mg of dry matter from the sample was placed in the filter paper bag, and then, the filter paper bag was placed in a Soxhlet extractor and extracted with pure water for 6 h. At the end of the extraction, the filter paper bags were dried to a constant weight and then extracted with absolute ethanol for 6 h. At the end of the extraction, the filter paper bag was dried to a constant weight and weighed. This weight was denoted m1 (unit: mg). Then, 300 mg of the dry matter from the extracted sample was placed in a test tube; 3 mL of 72% sulfuric acid was added and mixed well, and the mixture was shaken every 10 min in a water bath at 30 °C for 1 h. Subsequently, 84 mL of pure water was added to the test tube, and the tube was tightly sealed with a lid. After being sterilized at 121 °C for 1 h and cooled to room temperature, the supernatant was centrifuged and filtered with a 0.22 $\mu$m filter membrane. A part of the liquid was used for liquid phase determination of glucose and xylose contents and calculation of cellulose and hemicellulose contents. The cellulose and hemicellulose contents were calculated using the following formula

$$\text{the content of cellulose (\%)} = \frac{c_1 \times V \times 0.9}{m} \times 100 \quad (1)$$

$$\text{the content of hemicellulose (\%)} = \frac{c_2 \times V \times 0.88}{m} \times 100 \quad (2)$$

The absorbance value ($A$) of the other portion of liquid was measured at 320 nm. The above precipitates were washed with hot water and weighed to record the precipitate mass $m_2$ (unit: mg). The precipitate was combusted in a muffle furnace at 550 °C for 4 h, and the ash mass ($m_3$, unit: mg) was determined.

$$\text{the content of acid lignin (\%)} = \frac{A_{320} \times V \times (m_1 - m_0)}{\varepsilon \times m \times m_t} \times 100 \quad (3)$$

$$\text{the content of insoluble lignin (\%)} = \frac{(m_2 - m_3) \times (m_1 - m_0)}{m \times m_t} \times 100 \quad (4)$$

$$\text{the content of lignin (\%)} = \text{acid lignin (\%)} + \text{insoluble lignin (\%)} \quad (5)$$

Note:
$c_1$: glucose concentration, mg/mL;
$c_2$: xylose concentration, mg/mL;
$V$: total reaction volume, 87 mL;
0.9: conversion of glucose to cellulose;
0.88: conversion of xylose to hemicellulose;
$\varepsilon$: absorption rate of acid-soluble lignin at 320 nm, 25 L/(g·cm);
$m$: dry matter mass of the sample to be measured, 300 mg;
$m_0$: filter paper bag mass, mg;
$m_1$: mass after extraction, mg;
$m_2$: precipitate mass, mg;
$m_3$: mass of ash, mg;
$m_t$: initial mass: 1000 mg.

**2.4. Metagenomic Sequencing.** Based on identical dry weights, three groups of parallel samples from each experiment were evenly mixed and sent to Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) for metagenomic sequencing. We used Illumina HiSeq sequencing during metagenomic sequencing, and the reagents and methods were consistent with those in previous studies.[21] The original data obtained after sequencing are stored at NCBI (https://www.ncbi.nlm.nih.gov/) under accession number SRP364545.

Similarly, the method for gene prediction was consistent with that in previous studies,[21] but the removed host DNA sequences needed to be changed to the following sequences:

(1) https://www.ncbi.nlm.nih.gov/genome/46640
(2) https://www.ncbi.nlm.nih.gov/genome/?term=Scutellaria+baicalensis
(3) https://www.ncbi.nlm.nih.gov/genome/?term=txid1238147
(4) https://www.ncbi.nlm.nih.gov/genome/?term=txid222567

After host genes were removed, a new gene set was created according to the CAZy database (http://www.cazy.org/), and then BLASTP was used to compare the nonredundant gene set with the NR database (e-value ≤1 × $10^{-5}$). The species annotation results related to the CAZyme function were obtained from the corresponding taxonomic information database in the NR database. Then, the relative abundance of CAZyme-related species was calculated by using the sum of gene abundances at the genus level. Hummscan (https://www.ebi.ac.uk/Tools/hmmer/) was used to conduct a BLAST search for a new gene set with the CAZy database (e-value ≤1 × $10^{-5}$) to obtain CAZyme annotation information. The relative abundance of CAZymes was calculated from the sum of the gene abundances of CAZymes.

**2.5. Data Analysis.** We used Python 3.7 (Scipy module) to calculate Spearman's correlation coefficients between the factors, and Cytoscape 3.7.2 was employed to visualize the correlation coefficients. We used SPSS 19.0 to conduct the ANOVA significance tests. R 4.1.3 (vegan and ade4 packages) was used for Shannon index and principal coordinate analysis (PCoA). Origin 2021 was used to plot data. The specific parameter settings were consistent with those in previous studies.[21]

## 3. RESULTS AND DISCUSSION

**3.1. Changes in Physical and Chemical Indexes.** The temperature curves for groups CK and $T$ were different (Figure 2a) during CMR composting. The compost temperature of group $T$ increased significantly ($p < 0.05$), reaching 67.2 °C on Day 1 and reaching the maximum temperature (71.0 °C) on Day 2. In addition, the temperature in group $T$ remained at 70 °C for 2 days and above 60 °C for 7 days. Wang et al. also studied the thermophilic composting of CMR.[22] In their study, the highest temperature reached was also 70 °C, but the temperature only remained above 60 °C for 5 days, which was shorter than the duration in this experiment. In this study, the temperature of group CK also increased significantly on Day 1 ($p < 0.05$), reaching 60.9 °C, which was 6.3 °C lower than that


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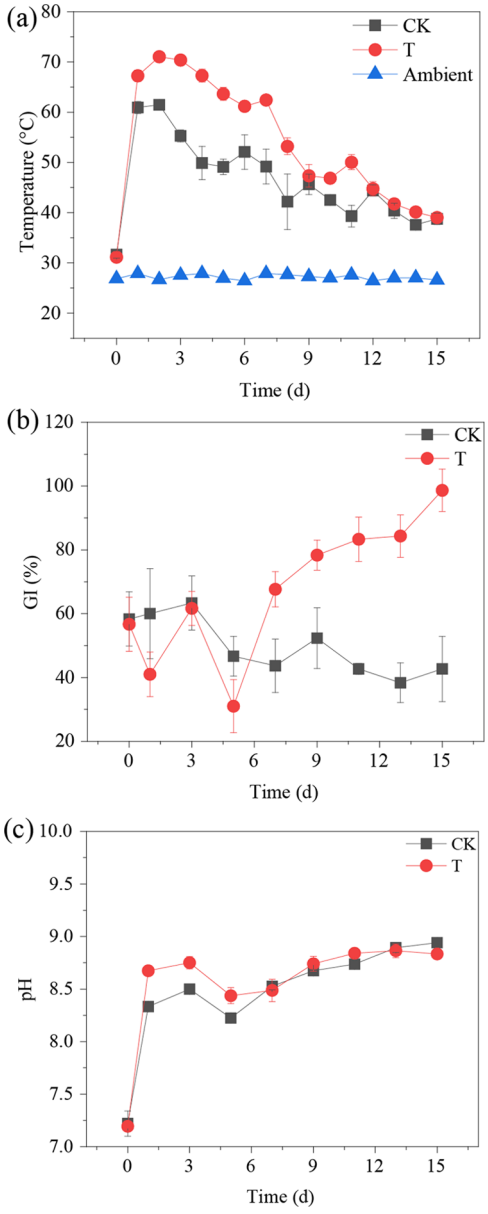


**Figure 2.** Variations in temperature (a), GI (b), and pH (c) during Chinese medicine residue composting.

of group *T*. Subsequently, the temperature of the CK group reached a maximum of 61.5 °C on Day 2, which was 9.6 °C lower than that of group *T* ($p < 0.05$). The CK group maintained a temperature above 60 °C for only 2 days, and composting efficiency was poor, which may have been caused by the high fiber content of the CMR that was difficult for local microorganisms to degrade and utilize. Therefore, the thermophilic microbial agents used in this study not only significantly increased the temperature of CMR composting but also prolonged the thermophilic period during composting.

The seed germination index (GI) can reflect the degree of maturation of a compost. As shown in Figure 2b, during CMR composting, the initial GI value of the compost in the two groups of experiments was not significantly different, at 58.3% (CK) and 56.7% (*T*). After 15 days of composting, the GI of group *T* increased significantly ($p < 0.05$), while that of group CK decreased gradually. The GI of group *T* reached 98.7% on day 15, reaching the compost maturation standard (GI ≥ 85%). After composting, the GI of group CK was only 42.7, 56.7% lower than that of group *T*, and it did not reach the compost maturation standard. In a study by Wang et al., it took 35 days for an herb residue compost to reach the maturity threshold,[22] which was longer than that of group *T* in this study. This may have occurred because multiple microorganisms were added as thermophilic microbial agents in this study.

The pH changes in the compost in groups *T* and CK were similar (Figure 2c). The pH values of both experimental groups were found to increase, which may be attributed to the production of alkaline substances, such as ammonia, resulting from the microbial decomposition of nitrogen-containing organic matter.

**3.2. Changes in Crude Fiber Content.** As shown in Figure 3, the residue contained 17.7% cellulose, 8.4% hemicellulose, and 55.2% lignin, accounting for 81.2% of the dry matter content. Lignin accounted for the highest proportion of the residue, at 67.9% of the crude fiber content, and the remaining 32.1% was cellulose and hemicellulose. The crude fiber content of CMR is relatively high, but the presence of large amounts of crude fiber impedes effective composting.[5] Therefore, it is important to study the degradation of crude fiber during the composting process.

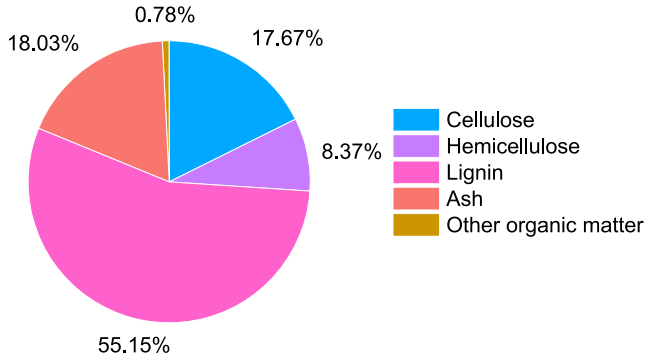


**Figure 3.** Content of each component in Chinese medicine residue.

As shown in Figure 4a, during CMR composting, the cellulose content of the two groups showed similar changes and exhibited downward trends. After 15 days of composting, the cellulose content of group CK decreased to 3.8% and that of group *T* decreased to 3.0%. Compared with the initial value, the cellulose content of group *T* was reduced by 83.3%, which was 5.1% more than that of group CK, but the difference was not significant ($p \geq 0.05$). During composting, the cellulose content of the two groups began to show a difference on Day 3 ($p < 0.05$), and the difference remained until Day 7. Xu et al. showed that increasing the temperature of compost and extending the thermophilic period could effectively improve crude fiber degradation.[23] In this study, within 3 to 7 days of CMR composting, the average temperature difference between the two groups of experiments reached 13.9 °C. This may have been caused by the different composting temperatures in the two groups of experiments, which led to different cellulose degradation rates. In a study by Bi et al., the cellulose content of CMR was as high as 39%, and the degradation rate was as high as 63% after 40 days of composting.[24] The cellulose degradation rates of the two groups were higher than those reported by Jingfang et al., mainly because the cellulose content of the CMR used in this experiment was lower and the


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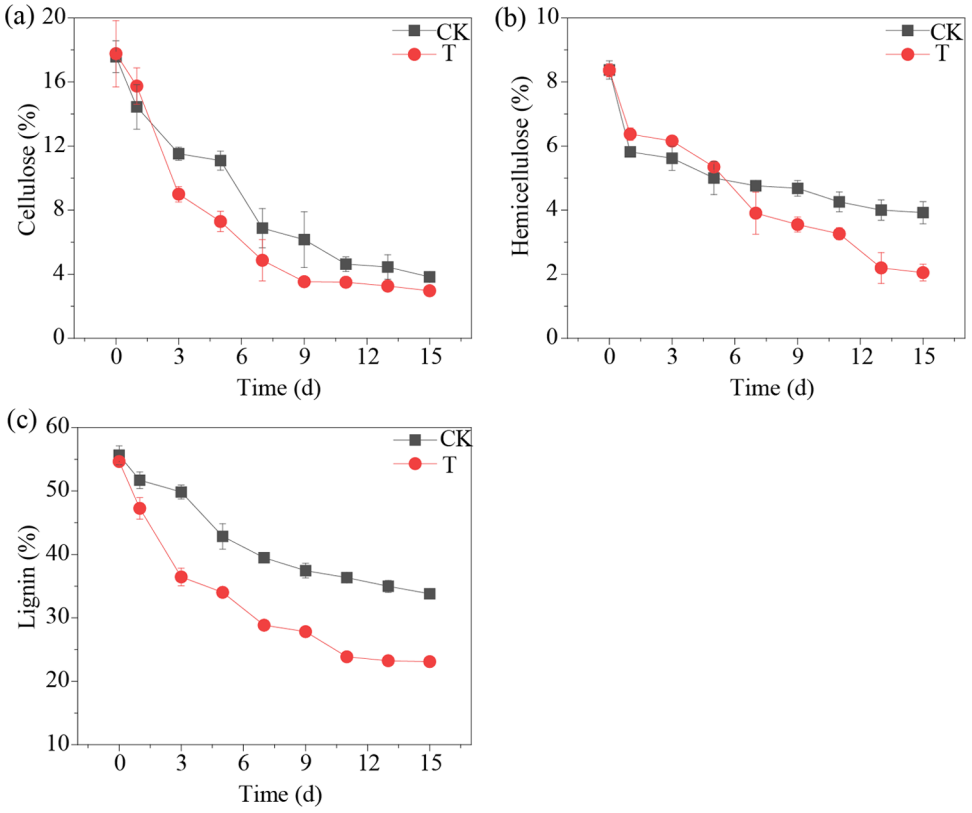


**Figure 4.** Changes in the content of cellulose (a), hemicellulose (b), and lignin (c) during Chinese medicine residue composting.

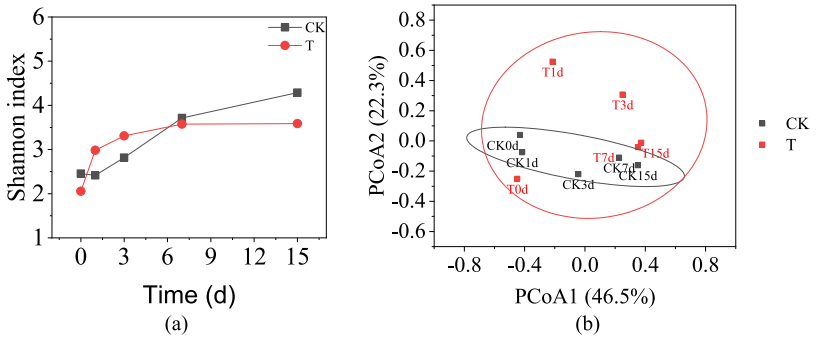


**Figure 5.** (a) Shannon index of CAZyme-related microbes and (b) PCoA of CAZyme-related bacterial communities. The dots represent samples taken in different composting periods.

average composting temperature in the two groups of experiments was higher than that previously reported. Therefore, CMR degradation by the thermophilic bacterial agents used in this study was better than that in a previous report.

As shown in Figure 4b, the hemicellulose content of the two groups showed similar changes during CMR composting, and both showed a downward trend. After 15 days of composting, the hemicellulose content in group CK and group *T* decreased to 3.9 and 2.1%, respectively. Compared with the initial value, the hemicellulose content in group *T* was reduced by 75.5%, which was 22.5% more than that in group CK, and degradation was significant ($p < 0.05$). The hemicellulose content in group *T* was lower than that in group CK on Day 9, but the difference was not significant until Day 13 ($p < 0.05$). Wang et al. found that after different microbial agents were added, the initial microbial communities in CMR compost changed only slightly, but after the thermophilic temperature stage, the microbial community structure of different experimental groups showed significant changes.[22] In this study, after thermophilic microbial agents were added to group *T*, the structure of the microbial communities changed after the thermophilic stage, which may have been the reason for the inconsistent degradation of hemicellulose in the CMR.

As shown in Figure 4c, the lignin content of the two groups showed similar changes, with a downward trend. After 15 days of composting, the lignin content of group CK decreased to 33.4% and that of group *T* decreased to 23.1%, and the resulting degradation was significant ($p < 0.05$) in the two treatments ($p < 0.05$). The lignin content in group *T* was significantly lower than that in group CK on the first day of composting ($p < 0.05$). On Day 1, group *T* was in the thermophilic stage, and the temperature reached 67.2 °C. Xu et al. found that when the temperature remained above 60 °C for 15 days during the co-composting process of cow manure and rice straw, the lignin degradation rate was close to 30%.[23]



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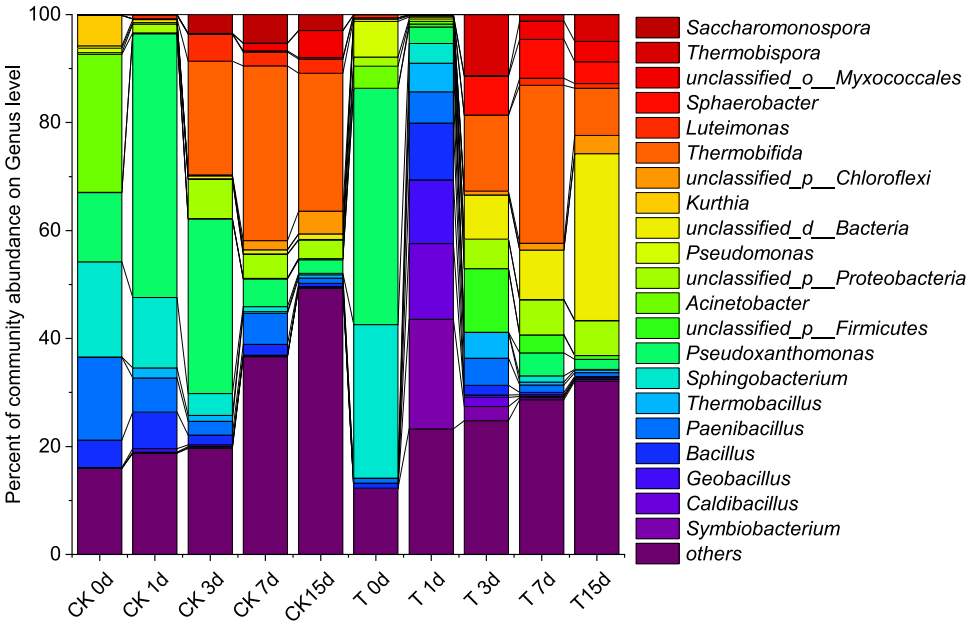


**Figure 6.** Relative abundance of CAZyme-related bacteria at the genus level during Chinese medicine residue composting.

When the temperature was below 50 °C for 15 days, the degradation rate of lignin was only approximately 10%. Similarly, in this study, the degradation rate of lignin in group *T* was not inhibited under high-temperature conditions, which was similar to the results of Jie et al., indicating that a high temperature could accelerate the degradation of lignin.

**3.3. Changes in CAZyme-Related Microbial Communities.** *α* diversity analysis can reflect the number of species in a microbial population and can be used to estimate the abundance and diversity of a bacterial community through Shannon index calculations. The larger the Shannon index is, the greater the bacterial community diversity.[25] As shown in Figure 5, to better understand the composition and structure of the compost community, the Shannon index of samples was calculated after metagenomic sequencing, and a difference was found in the *α* diversity between group CK and group *T*. Additionally, the *α* diversity of both groups increased with time. The *α* diversity in group *T* increased rapidly, exceeding that in group CK on Day 1, and then increased slowly. However, the *α* diversity of group CK began to increase slowly and only exceeded that of group *T* during the late composting stage, indicating that there were more CAZyme-related microbial species in group *T* than in group CK during the early composting stage.

CAZyme-related microorganisms are those capable of expressing CAZyme-related genes. According to PCoA (Figure 5b), PC1 and PC2 accounted for 68.9% of the total variance in the microbial communities. During different composting stages, the composting temperatures of the two groups were different, and their CAZyme-related microbial communities were significantly separated, indicating that the addition of bactericides and changes in composting temperature could change the CAZyme-related microbial communities, which has also been confirmed in previous studies.[26]

In the early stages of composting, the CAZyme-related microbial communities in the CK group were similar on Day 0 and Day 1, and the microbial communities did not change significantly until Day 3. The CAZyme-related microbial communities in group *T* were different from those in group CK on Day 0. Figure 6 shows the distribution of the CAZyme-related microbial communities at the genus level. On Day 0, the three dominant CAZyme-related bacteria in group CK were *Acinetobacter*, *Sphingobacterium*, and *Paenibacillus*, and the three CAZyme-related dominant bacteria in group *T* were *Sphingobacterium*, *Pseudomonas* and *Pseudoxanthomonas*, which were different from those in group CK, indicating that there were many CAZyme-related microorganisms in the added thermophilic bacteria, which had a certain effect on the community structure of CAZyme-related microorganisms in the CMR. However, during composting, the relative abundance of the bacteria initially dominant in both groups decreased with increasing time, which indicated that these microorganisms were not favored during CMR composting. In addition, *Geobacillus* and *C. yamamurae* were barely detectable as CAZyme-related microorganisms on Day 0. It is possible that the abundance of these two microorganisms in the compost was so low that they could not be detected in the initial stage, as they constitute only a small proportion of the total microbial population in the compost. According to Figure 6, the CAZyme-related microbial communities in group *T* were significantly different on Day 0 and Day 1. On Day 1, the dominant bacteria in group *T* changed into *Symbiobacterium*, *Geobacillus*, and *Caldibacillus* (Figure 6). These three types of bacteria can grow at high temperatures,[27−29] indicating that an increase in composting temperature can significantly affect CAZyme-related microbial communities. During the middle and late composting periods, the CAZyme-related microbial community compositions began to converge on Days 7 and 15 in group CK, while the CAZyme-related microbial communities essentially stabilized on Days 7 and 15 in group *T*, indicating that the composting temperature decreased and that the CAZyme-related microbial communities began to stabilize.

As shown in Figure 6, on Day 7 and Day 15, the beneficial CAZyme-related bacteria in the CK group was *Thermobifida*. In group *T*, the dominant CAZymes belonged to *Thermobifida* and *unclassified_d_Bacteria*, which were different from those in group CK. Therefore, the microbial communities in the two groups were different during different periods, which resulted


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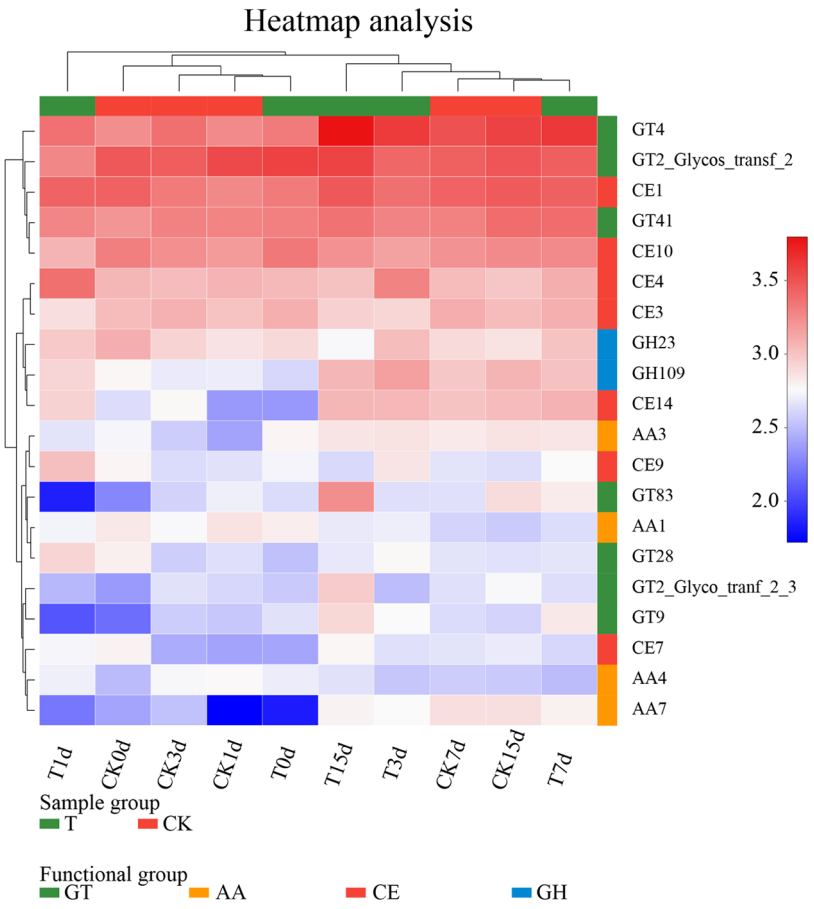


**Figure 7.** Gene expression abundances of CAZymes at the family level during Chinese medicine residue composting. Only the top 20 gene expression abundances are shown.

in the different degradation rates of crude fiber in the two groups. The dominant CAZyme-related bacteria that were enriched using rice straw by Wang et al. also contained *Thermobifida*, but their abundance was relatively low,[8] which is slightly different from the results in this study, and this might be due to the use of different substrates.

**3.4. Changes in the Relative Abundance of CAZyme-Related Genes.** The degradation of cellulose, hemicellulose, and lignin was closely related to the CAZymes secreted by microorganisms. These CAZymes can degrade, modify, and generate glycosidic bonds. The results of metagenomic sequencing were annotated by the CAZy database, and 478 kinds of CAZymes were obtained: 242 kinds of glycoside hydrolases (GHs), 91 kinds of glycosyl transferases (GTs), 62 kinds of polysaccharide lyases (PLs), 48 kinds of carbohydrate-binding modules (CBMs), 16 kinds of carbohydrate esterases (CEs), and 19 types of auxiliary activities (AAs). Figure 7 shows the top 20 kinds of CAZymes based on abundance during the composting of CMR, including 2 kinds of GHs, 7 kinds of GTs, 4 kinds of AAs, and 7 kinds of CEs. Table S1 shows the descriptions of the top 20 kinds of CAZymes.

As shown in Figure 7, cluster analysis was performed for CAZyme functional levels on the samples, and the CAZyme function of T0d was similar to that of CK0d, CK 1d, and CK 3d, indicating that the addition of thermophilic bacteria had little effect on the initial CAZyme function of the CMR. In addition, compared with T0d, the CAZyme gene expression level of T1d significantly differed, while CK0d showed little difference from CK 1d and CK 3d. These results indicated that the change in temperature had a substantial effect on the expression of CAZyme genes during the process of thermophilic composting of CMR. After composting, the relative abundances of CAZyme-related genes in CK15d and T15d were significantly different. The results showed that the two experimental groups retained differences in the function of CAZymes when the temperature dropped to the same value during the late composting stage. This difference in function may be one of the reasons for the difference in the degree of cellulose, hemicellulose, and lignin degradation between the two groups.

Most cellulases and hemicellulases are GHs. GH23 and GH109 were the main GH families detected during the composting of CMR. The GH23 family members include G-type lysozyme (EC 3.2.1.17) and peptidoglycan lyase (EC 4.2.2.n1), which function by slicing glycosidic bonds through a substrate-assisted mechanism that is independent of water.[8] However, one member of the GH109 family is $\alpha$-nitrogen-acetylgalactosaminase (EC 3.2.1.49), which is mainly involved in metabolic pathways related to starch synthesis and transformation.[8] Wang et al. found that the abundance of the CH23 family was the highest among the bacteria enriched using rice straw, and it was related to the degradation of fiber-derived substances.[8] Therefore, GH23 may be involved in the degradation of cellulose and hemicellulose in the CMR in this study. Based on Figure 7, the total expression level of the GH23 family genes in group *T* was slightly higher than that in group CK, but it was not significant, which may have occurred because the differential expression of the other families of


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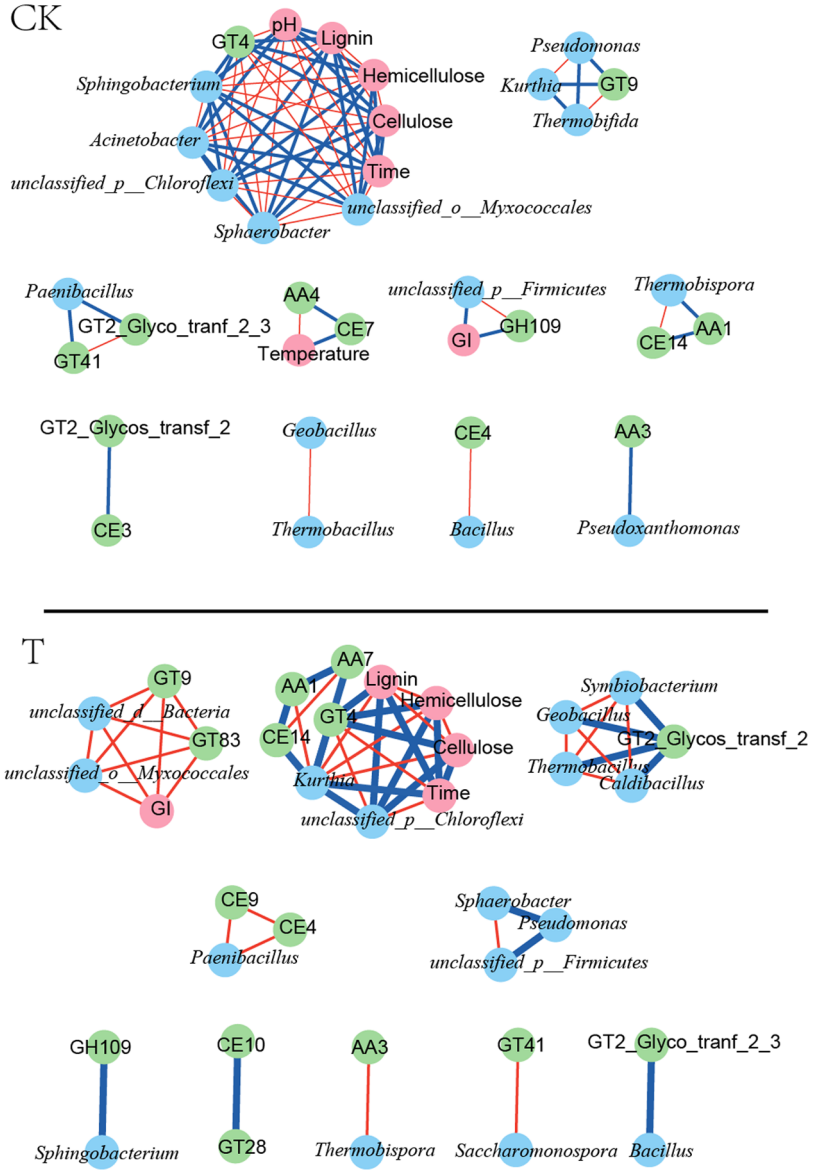


**Figure 8.** Network analysis showing the connections among CAZymes (green), CAZyme-related bacterial communities (blue), and environmental factors (pink). The red solid line represents a significant positive correlation ($r \geq 0.9$, $p < 0.05$), and the blue solid line represents a significant negative correlation ($r \leq -0.9$, $p < 0.05$). The thickness of the solid line represents the absolute value of the correlation coefficient.

genes led to the inconsistent degradation of cellulose and hemicellulose in the two groups.

The relative abundance of GTs was higher during the composting of CMR, and 7 of the 20 most abundant CAZymes were GTs, among which the GT4 family had the highest relative abundance, followed by the GT2_Glycos_transf_2 family. These two families of CAZymes can catalyze a variety of reactions, including the removal of glycogroups and the phosphorylation of glycosidic bonds.[30] The relative abundance of the GT4 family genes in both groups continued to increase during composting. After composting, the relative abundance of the GT4 family genes in group CK was 2.2 times that of CK0d, while that of the GT4 family genes in group *T* was 2.9 times that of T0d, which was higher than that in group CK. In addition, the relative abundance of T15d was 1.6 times that of CK15d. For the GT2_Glycos_transf_2 family of genes, the relative abundance of T15d was 1.2 times that of CK15d. The GT4 and GT2_Glycos_transf_2 families were highly expressed in group *T*, which may be one of the reasons for the distinct degradation of crude fiber in group *T*.

Most lignin-degrading enzymes belong to AAs.[31] AA3 and AA1 were the main AAs during the composting of CMR. Wang et al. reported that AA2 was the main lignin-degrading enzyme in rice straw,[8] which is different from the results in this study, possibly due to differences in the lignin structures of tea stalk and rice straw. The AA3 family genes encode a variety of enzymes, including cellulose disaccharide dehydrogenase, which assists in lignin degradation as well as in the metabolic activities of other AA and GH families.[32] Therefore, AA3 plays an important role in lignin degradation. In addition, the AA1 family genes encode laccase, which also plays an important role in lignin degradation. During composting, the total relative abundance of the AA1 family genes was similar between the two groups, but the total relative abundance of the AA3 family genes was 24.8% higher in group *T* than in group CK. Therefore, it is preliminarily speculated that the lignin


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degradation rate of group *T* was higher than that of group CK, possibly due to the high AA3 gene expression level of group *T*.

The CE family genes promote the role of the CH family mainly by encoding carbohydrate esterase for ester hydrolysis.[33] During the composting of CMR, CE1 and CE10 were the main CE families. The CE1 family genes encode feruloylesterase, which plays an important role in lignin degradation.[33] CE10 has isoenzymes similar to those of the CE1 family, but the enzymes of CE10 are mainly for noncarbohydrate substrates.[34] During the composting of CMR, the relative abundances of the CE1 and CE10 family genes were higher in both groups, but there was no significant difference. It was preliminarily speculated that the change in the compost temperature had little effect on the expression of CE genes. Therefore, the expression of the CE family of genes did not lead to an improvement in the degradation of lignin in group *T* relative to that in group CK.

According to the above results, the addition of thermophilic microbial agents did not affect the initial CAZyme function of the compost but significantly changed the expression of functional CAZyme genes by influencing the change in the compost temperature. The degradation efficacy of crude fiber in group *T* was better than that in group CK during composting, which might be related to the high relative abundances of the GT4, GT2_Glycos_transf_2, and AA3 genes.

**3.5. Correlation Network Analysis.** Network analysis was used to evaluate the effects of the CAZyme genes, CAZyme-related bacteria, and environmental factors on the degradation of crude fiber during composting (Figure 8 and Table 3). The

**Table 3. Statistical Results of Network Correlation Analysis**

| parameters | CK | *T* |
|---|---|---|
| nodes | 35 | 36 |
| edges | 77 | 58 |
| average number of neighbors | 4.4 | 4.4 |
| length of characteristic path | 1.00 | 1.04 |
| network density | 0.07 | 0.05 |

network of group CK consisted of 35 nodes and 77 edges, and the network of group *T* consisted of 36 nodes and 58 edges. In addition, the network characteristic path length of group CK was 1.0, the average number of neighbors was 4.4, and the network density was 0.07. The network characteristic path length of group *T* was 1.04, the average number of neighbors was 3.22, and the network density was 0.05. After thermophilic microbial agents were added, the number of nodes related to crude fiber degradation in group *T* was 1 more than that in group CK, but the average number of neighbors decreased by 25.0%, the length of the characteristic path increased by 4.0%, and the network density decreased by 28.6%. This indicates that group CK had a more compact network, while group *T* had a looser network. Therefore, we speculated that the increased crude fiber degradation ability of group *T* might be due to fewer restrictions in the degradation process.

As shown in Figure 8, a significant positive correlation existed among cellulose, hemicellulose, and lignin in the CK group. Their content showed significant negative correlations with time, pH, GT4, *unclassified_p_Chloroflexi*, *Sphaerobacter*, and *unclassified_o_Myxococcales*. The longer the composting time was, the more evident the degradation of crude fiber. During composting, an increase in pH can promote the degradation of the three classes of crude fiber. Researchers have reported that the addition of cellulose-degrading bacteria alone has no significant effect on an increase in reactor pH.[35] In addition, an increase in compost pH is mainly caused by the degradation of nitrogen-containing organic matter.[36] According to Niu et al., crude fiber-degrading bacteria showed high degradation activity in a pH 7−9 environment.[37] Therefore, as the pH increased, it provided a good living environment for crude fiber-degrading bacteria and promoted crude fiber degradation in the CMR. In addition, the increase in GT4 content also promoted the degradation of the three classes of crude fiber. Researchers have reported that 50% of all glycosyl transferases belong to the GT4 and GT2 families, which catalyze a variety of reactions, including some key steps in the n-glycosylation pathway.[30] Therefore, the GT4 family plays an important role in the degradation of crude fiber during the composting of CMR.

During the process of crude fiber degradation in group CK, the three CAZyme-related bacteria *unclassified_p_Chloroflexi*, *unclassified_o_Myxococcales*, and *Sphaerobacter* played dominant roles. *Chloroflexi* has been reported to be prevalent in a variety of ecosystems and to contain many CAZymes. In addition, some *Chloroflexi*, such as *Ktedonobacteria*, show greater degradation capacity for crude fiber.[38] There are few direct reports on the degradation of crude fiber by *Myxococcales*, but the relative abundance of *Myxococcales* is higher in composts rich in crude fiber.[39,40] Therefore, it is reasonable to speculate that *Myxococcales* also plays a role in the degradation of crude fiber. *Sphaerobacter* has also been reported in compost rich in crude fiber.[41,42] Therefore, *unclassified_p_Chloroflexi*, *unclassified_o_Myxococcales*, and *Sphaerobacter* play a role in crude fiber degradation during the composting of CMR.

As shown in Figure 8, in group *T*, there was also a significant positive correlation among cellulose, hemicellulose, and lignin. These compounds showed significant negative correlations with time, GT4, and *unclassified_p_Chloroflexi*. After thermophilic microbial agents were added, the composting temperature in group *T* changed dramatically, inducing significant changes in the microbial community structure, which made the correlations among *Sphaerobacter*, *unclassified_o_Myxococcales*, and the degradation of crude fiber not significant. In the CK group, *Unclassified_o_Myxococcales* and *Sphaerobacter* played a significant role in promoting the degradation of crude fiber. *Unclassified_o_Myxococcales* in group *T* reached a stable state on Day 7, and its content was 3.4%, while that in group CK was only 1.4%. Therefore, *Unclassified_o_Myxococcales* contributed to crude fiber degradation in group *T*. The average relative abundance of *Sphaerobacter* was 0.1% in group CK but increased to 3.7% in group *T*, although the changes in *Sphaerobacter* were not significantly correlated with the degradation of crude fiber. It has been reported that *Sphaerobacter* is abundant in the high-temperature and maturation stages of composting.[42] *Sphaerobacter* growth was favored in the group *T* compost due to the group's high compost temperature and greater postcompost maturity than group CK, which promoted the degradation of crude fiber in group *T*. Therefore, the thermophilic microbial agents indirectly promoted the growth of *unclassified_o_Myxococcales* and *Sphaerobacter*, thereby promoting the degradation of crude fiber.

As shown in Figure 8, in the CK group, *Acinetobacter* and *Sphingobacterium* were positively correlated with crude fiber


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content. In group *T*, the content of *Acinetobacter*, *Sphingobacterium*, and crude fiber became insignificant. *Acinetobacter* has been reported to be involved in lignin degradation,[43] but a variety of pathogenic bacteria are also present.[44] *Acinetobacter* was not considered suitable for survival in compost environments, and these bacteria may compete with some lignin-degrading bacteria. The mean relative abundance of *Acinetobacter* was 5.2% in CK and only 0.9% in group *T*. The temperature of the compost in group *T* was higher than that in group CK; it is possible that the high temperature killed most of the *Acinetobacter* that competed with lignin-degrading bacteria, thereby improving the degradation of lignin in group *T*. In addition, *Sphingobacterium* is not conducive to the degradation of crude fiber,[45] and its existence also impedes the degradation of crude fiber in CMR. The average relative abundance of *Sphingobacterium* in group CK was 11.6% over the first 3 days of composting, while the relative abundance of *Sphingobacterium* in group *T* decreased from 28.4% to 3.6% on Day 1 of composting and was almost 0% on Day 3 of composting, which showed that the high temperature in group *T* effectively inactivated *Sphingobacterium*. Thus, *Sphingobacterium* could not impede the degradation of crude fiber in group *T*. Therefore, thermophilic composting of CMR leads to the effective inactivation of *Acinetobacter* and *Sphingobacterium* and promotes the degradation of crude fiber.

## 4. CONCLUSIONS

The effect of thermophilic microbial agents on the degradation of crude fiber during the composting of CMR was studied. The results showed that the composting temperature of the CMR could be increased to 71.0 °C and the composting time could be shortened to 15 days with the addition of thermophilic microbial agents. The degradation rates of cellulose, hemicellulose, and lignin increased to 83.3, 75.5, and 57.7%, respectively. The expressions of GT4, GT2_Glycos_transf_2, and AA3 in the compost were significantly increased after the addition of the thermophilic microbial agents, and this was one of the main reasons for the promotion in the degradation of crude fiber in the CMR. In addition, thermophilic microbial agents changed CAZyme-related microbial communities, inducing the relative abundances of *unclassified_o_Myxococcales* and *Sphaerobacter* to increase and the relative abundances of *Acinetobacter* and *Sphingobacterium* to decrease. Therefore, thermophilic microbial agents have broad application potential in promoting the degradation of crude fiber during the composting of CMR.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c05442.

Descriptions of CAZymes (top 20) (PDF)

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### Notes

The authors declare no competing financial interest.

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