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Dynamic Changes in Physicochemical Properties and Microbial Community in Three Types of Recycled Manure Solids for Dairy Heifers

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Abstract: Recycled manure solids (RMSs) are widely utilised as beddings due to their economic and environmentally friendly features. Internal change in RMSs plays a vital role in the stable operation and management of beddings. However, the internal microenvironment of various manure beddings has not been fully reported. Therefore, we evaluated the physicochemical properties, internal gases and changes in the microbial community of the in situ fermentation beds, which were prefermented by cow manure with sawdust (FSD), straw (FST) and sawdust–straw mixture (FM), at a farm in Jiangsu, China, from June to September 2022. The results indicated that the FSD and FM beds were more capable of degrading organic matter (OM), accumulating total nitrogen and processing a more stable pH environment. FSD bed promoted the conversion of nitrate–nitrogen and ammonium–nitrogen (NH_4^+-N). Different treatments and times had significant effects on bacterial and fungal communities. FSD enriched *Chloroflexi*, and FST enriched *Actinobacteriota* in the early stage, while FM enriched *Proteobacteria* in the late stage. Bacterial communities were more sensitive to NH_4^+-N and OM, while fungal communities were more sensitive to temperature and pH. FSD had potential advantages concerning N conversion and C emission reduction. The results of the study revealed the microenvironmental dynamics during bedding use, providing a theoretical basis for the use of a compost bedding system for managing recycled dairy manure.

Keywords: cow manure; biowaste bedding material; environmental factors; microbial community

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1. Introduction

The rapid development of large-scale agriculture produces agricultural by-products as well as large quantities of organic waste, such as straw and manure. Annual production of cow manure in China has reached about 1.38 billion tones [1], and annual production of straw can reach about 900 million tons [2]. In forest ecosystems, wood processing produces much sawdust, and about 50% of harvested timber ends up as waste [3]. Accumulation and improper disposal of livestock manure, sawdust and straw can lead to atmospheric, soil and water pollution [4,5]. Therefore, an appropriate approach to managing cow manure, sawdust and straw waste is urgently needed.

As a strategy for manure treatment, composted or freshly recycled manure solids (RMSs) bedding is proposed and widely used in several regions such as the United Kingdom, Canada and other regions due to its economy, availability and cow comfort [6–8]. According to the different production methods of RMSs, it can be roughly divided into three modes [9], including direct use after solid–liquid separation, anaerobic fermentation and aerobic fermentation. Aerobic fermentation is essentially a biodegradation process, which is driven by a variety of microbial communities interacting and synergising with each other to decompose and transform compost organic matter [10]. Bacteria and fungi dominate the composting system and also play an important role in the process of composting [11].

Bacteria, which are present in huge numbers during all periods of composting, are able to act as decomposing transmuters of most of the organic matter in the pile [12], and fungi could break down complex polymers present in the compost [13]. During the process of aerobic fermentation to produce RMSs, the bed fermentation temperature will reach 50 °C for more than 5 days to destroy pathogens and increase the safety of the litter [14]. Previous studies have shown that the application of agricultural and forestry by-products such as straw, corn stalks and sawdust is an effective way to accelerate compost maturation through exogenous C additives, which can support microbial metabolism and accelerate compost maturation [15,16]. Therefore, in this experiment, sawdust, wheat straw and cow manure were mixed in different proportions for aerobic fermentation to produce bedding.

Most previous studies on RMSs have focused on the physicochemical properties, safety and microbial composition during the preparation period, as well as animal welfare, barn environment and milk quality of RMS beddings during the feeding period [7,17–20]. In the actual culture stage, there are relatively few studies on the physicochemical properties, internal gas changes and the relationship between RMS bedding and microbial composition. Dynamic changes during the use period are crucial for fine padding management. Therefore, physicochemical analyses and high-throughput sequencing technology, internal microenvironment changes and key environmental factors, as well as the microbial community evolution in the FSD, FST and FM bedding, were studied by physicochemical analyses and high-throughput sequencing technology. The results of this study will provide a theoretical basis for fermentation bed management and cleaner production for cows.

2. Materials and Methods

2.1. Experimental Design and Animals

This experiment was carried out at a dairy farm in Jiangsu province, China, from June to September 2022 with permission from the Animal Management and Use Committee of Nanjing Agricultural University. Eighteen Holstein heifers (BW, 226.55 ± 4.61 kg; age, 6–7 months) were divided into three treatment groups at random, consisting of six individuals each: fermented sawdust (FSD), fermented straw (FST), and fermented sawdust–straw mixture (FM). For every group, there was 25.0 cm of bedding paved above the ground on the first day. The height of the bedding was tested by a steel ruler. Every pen had dimensions of 15.0 m × 5.0 m. A metal barrier that allowed animals in adjacent enclosures to come into contact with one another divided the pens. At the south of the barn, a drive-through alley with a concrete floor outside the pen allowed the tractor to distribute food. The space allowance per heifer was 12.5 m^2. The bed was tilled every six days with a rototiller. The heifers were fed ad libitum on a total mixed ration (TMR) and watered ad libitum. The diet was served twice daily at 07:40 and 14:40. The duration of the feeding experiment was 31 days.

2.2. Preparation of Bedding Materials

The RMS bedding was produced by aerobic fermentation of cow manure, added by sawdust and/or wheat straw. The cow manure was collected from the experimental dairy farm, and the wheat straw and sawdust were acquired near the farm. The sawdust, wheat straw and cow manure were combined into three heaps using the ratio shown in Table 1, and they were composted on a concrete surface in the dairy farm. Table 1 displays the initial characteristics of the compost ingredients. Every three days, a forklift was used to turn the piles after the temperature of the fermentation process had first risen beyond 55 °C. Every five days, the moisture content of the compost was measured. When the fermented temperature exceeded 50 °C for more than 7 days, and the moisture content dropped to 50%, the pile was stretched out to dry until the water content fell to about 30% [21,22]. The barn was then furnished with bedding supplies.

Table 1. Physical and chemical properties of bedding materials.

Items / Materials	Water Content (%)	Organic Matter (%)	Total N (%)	C/N Ratio	Bulk Density (kg/m$^{33.5}$)	Porosity (%)
Cow manure	81.43	69.12	1.97	20.38	—	—
Sawdust	13.94	79.43	0.26	176.52	—	—
Wheat straw	8.56	73.33	0.48	89.84	—	—
FSD [1]	67.89	76.11	1.01	43.87	267.10	61.85
FST [1]	67.3	71.91	1.16	36.03	294.47	63.58
FM [1]	67.02	67.25	1.05	37.10	334.27	61.40

[1] FSD, fermented sawdust (sawdust/cow manure = 3:1); FST, fermented straw (wheat straw/cow manure = 3:1); FM, fermented sawdust–straw mixture (sawdust/straw/cow manure = 2:1:1).

2.3. Bedding Material Sampling and Physicochemical Analysis

During the feeding experiment, bedding samples were collected every five days using a five-point sampling method, followed by mixing thoroughly and separating them equally into three parts. Then, samples were stored at 4 °C or −20 °C for physicochemical analysis. On d 1, 16 and 31, samples from each group were collected and snap-frozen in liquid nitrogen and then stored at −80 °C for microbial analysis. Bed temperature was measured at 9:00 and 15:00 every five days using a thermocouple thermometer at a depth of 15 cm at the sampling point of the bedding samples, and the five values obtained were averaged. The water content (WC) was analysed based on weight loss after drying at 105 °C through an oven (ESCO, Singapore) to a constant weight [23]. The pH and electrical conductivity (EC) were measured in a 1:10 (W/V) water extraction using a pH meter and a conductivity meter (INESA, Shanghai, China), respectively [24]. The organic carbon content was determined using the potassium dichromate oxidation method, organic matter (OM) = organic carbon × 1.724 [25]. Total nitrogen (TN) content was determined using the Kjeldahl method [26]. C/N ratio = organic carbon/total nitrogen. The nitrate–nitrogen (NO_3^--N) content was quantified using the method described in the agricultural industry standard of China organic fertiliser NY/T 1116–2014. The ammonium–nitrogen (NH_4^+-N) concentration was determined using the indophenol blue method [27]. The bulk density and porosity were determined using the method proposed by Ferraz et al. [28]. All measurements were conducted in triplicate.

2.4. Gas Concentration Measurement

The gases, including CO_2, CH_4, NH_3 and N_2O, were determined in triplicate every 5 days at 16:00 by a photoacoustic multi-gas monitor (INNOVA 1412, LumaSense Technologies SA, Ballerup, Denmark). Air samples were drawn from the 15 cm depth of each bed via the five-point sampling method [29].

2.5. DNA Extraction and Illumina MiSeq Sequencing of 16S rRNA and ITS1 Amplicons

Microbial DNA from a 0.5 g sample was extracted with a TGuide S96 Magnetic Soil/Stool DNA Kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. The quality and quantity of extracted DNA were determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Illumina MiSeq sequencing was employed to investigate the shifts of bacterial and fungal communities in composts. The V3–V4 region of the 16S rRNA gene was amplified using the primer set 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′), which produces accurate taxonomic information with little bias for different bacterial classes. The ITS1 gene was amplified using the primer set ITS 1F (5′-CTTGGTCATTTAGAG GAAGTAA-3′) and ITS 2 (5′-GCTGCGTTCTTCATCGATGC-3′), which assigns accurate taxonomic information for fungal classes. Both the forward and reverse 16S and ITS primers were tailed with sample-specific Illumina index sequences to allow for deep sequencing. After the individual quantification step, amplicons were pooled in equal amounts. For

constructed libraries, sequencing was performed using Illumina novaseq 6000 (Illumina, San Diego, CA, USA). The bioinformatics analysis of this study was performed with the aid of the BMKCloud (Biomarker Technologies Co., Ltd., Beijing, China).

2.6. Bioinformatic Analysis

According to the quality of a single nucleotide, raw data were primarily filtered by Trimmomatic (version 0.33). Identification and removal of primer sequences were processed by Cutadapt (version 1.9.1). Paired-end (PE) reads obtained from previous steps were assembled by USEARCH (version 10) and followed by chimera removal using UCHIME (version 8.1). The high-quality reads generated from the above steps were used in the following analysis. Sequences with similarity $\geq$ 97% were clustered into the same operational taxonomic unit (OTU) by USEARCH (v10.0), and the OTUs with reabundance < 0.005% were filtered. Taxonomy annotation of the OTUs was performed based on the Naive Bayes classifier in QIIME2 using the SILVA database (release 132) with a confidence threshold of 70%.

2.7. Statistical Analysis

All data are expressed as mean $\pm$ standard error of the mean (SEM). Statistical analysis was conducted using SPSS 26.0 software (SPSS Inc., Chicago, IL, USA). Bedding physicochemical properties and gas changes were analysed using one-way ANOVA. Multiple comparisons were made using Duncan's test to assess differences between any two groups. Alpha diversity (Chao 1, Shannon and Simpson indices) was calculated using QIIME2. Using the vegan and ade4 packages, Bray–Curtis distance-based PCoA (principal coordinate analysis) was carried out in R3.6.3, and intergroup similarity (ANOSIM) was analysed for both groups. The study employed linear discriminant research (LDA) effect size (LEfSe) research to uncover the noteworthy arrangement of plentiful modules across various samples. As a discriminative functional marker, the logarithmic LDA score was normalised to a size-effect threshold of 4.0 [30]. Mantel test was used to explore the relationship between the microbial community and bedding characteristics. Spearman correlation analyses were performed between the environmental factor and the relative abundance of microbes. Significance was declared at $p < 0.05$.

3. Results and Discussion

3.1. Changes in the Physicochemical Properties of the Bedding Material

Changes in the physicochemical properties of three groups during the process of bedding use are shown in Figure 1. Temperature significantly influences the growth rate and activity of microorganisms in compost [31]. The FST reached the highest temperature at d 16 (28.71 °C) and then gradually decreased, while the FM (32.75 °C) and the FSD (29.86 °C) reached the highest temperature at d 21 (Figure 1a). The temperature of the FST was higher than that of the FSD and the FM on d 16 ($p < 0.05$) and was significantly lower than that of the FSD and the FM from d 21 to 31 ($p < 0.001$). The higher temperatures in the FSD and FM at 21–31 days might be related to the presence of sawdust. Guo et al. [32] showed that treatments with the addition of sawdust were more suitable for microbial growth and activity and were able to accelerate the decomposition of organic matter and generate more heat.

Changes in OM reflect the rate of mineralisation and degradation of OM during the composting process. On the first day of the feeding experiment, the OM content was FSD > FM > FST ($p < 0.05$; Figure 1b), as all three bedding groups were pre-fermented and the use of compounds with high lignin content, such as sawdust, has been reported to reduce the loss of organic carbon during composting [33]. At the end of the experiment, the OM degradation rate was 4.10% for FSD and 8.65% for FM, while the OM of the FST increased by 16.22%. These results indicated that FSD and FM possessed a higher capacity to degrade OM in situ fermentation.

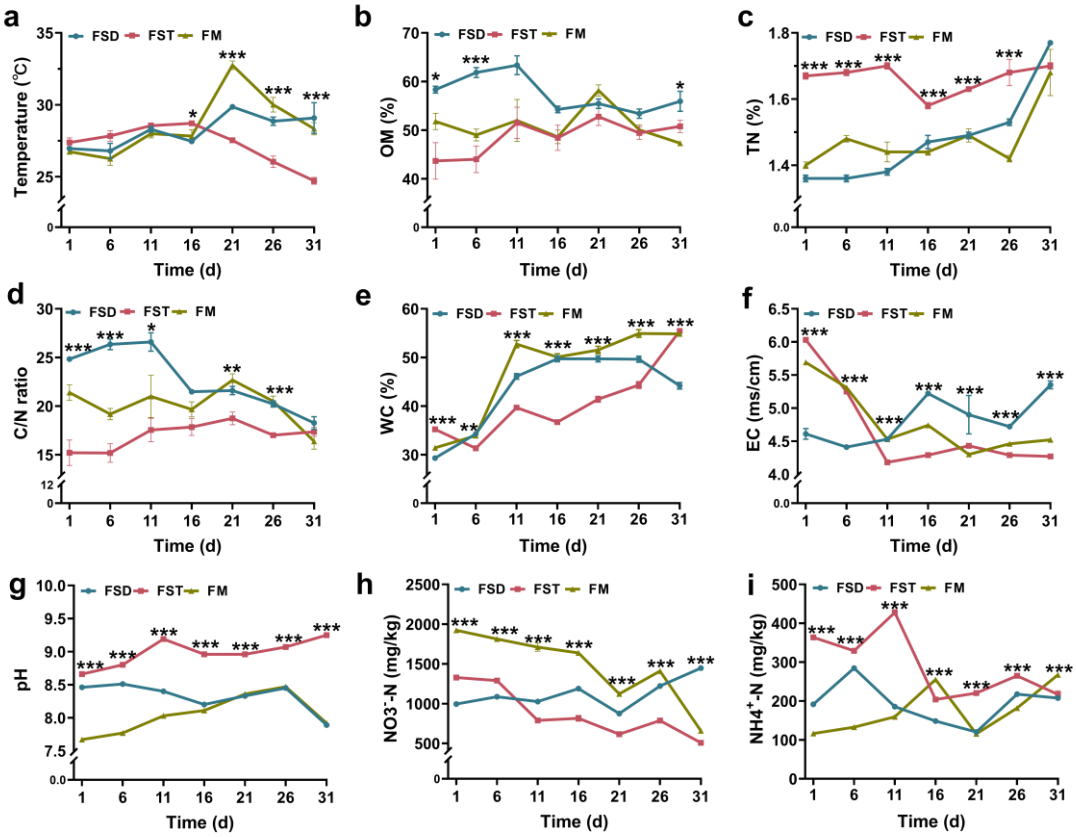

Figure 1. Dynamic changes in physicochemical properties of the bedding materials. Temperature (**a**), OM (**b**), TN (**c**), C/N ratio (**d**), WC (**e**), EC (**f**), pH (**g**), NO_3^--N (**h**) and NH_4^+-N (**i**) of the three groups. OM, organic matter; TN, total nitrogen; WC, water content; EC, electrical conductivity; NO_3^--N, nitrate–nitrogen and NH_4^+-N, ammonium–nitrogen. Data are shown as mean ± SEM, $n \geq 3$. FSD, fermented sawdust; FST, fermented straw; FM, fermented sawdust–straw mixture. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

TN content tended to be enhanced in all groups as the duration of compost mat use increased (Figure 1c). TN was significantly higher in the FST than that in the remaining two groups at d 1–26 ($p < 0.05$). TN levels usually increase as composting proceeds due to the mineralisation of organic matter, which reduces the weight of the compost and then results in a 'thickening effect' [34]. Additionally, the accumulation of excreta with longer feeding time can also lead to an increase in TN levels. At d 31, TN increased by 30.15% for FSD, 20.00% for FM and 1.80% for FST. This indicates that the usage of sawdust increased nitrogen retention in the bedding. Correlation analysis showed that TN was negatively correlated with C/N ratio, CO_2, CH_4, NH_3 and N_2O, and positively correlated with WC and NH_4^+-N ($p < 0.05$; Figure 6).

The C/N ratio is the most critical factor of efficient composting and is one of the most important factors affecting the microbial decomposition of organic matter [10]. The C/N ratio was significantly higher in FSD than in FST except on d 16 and 31 ($p < 0.05$; Figure 1d). A previous study suggested that when the C/N ratio in the bedding falls to 15, the old bedding needs to be removed and new bedding laid [35]. This is because, below this level of C/N ratio, fermentation in the bedding is inhibited and may result in higher nitrogen losses. All three groups had a C/N ratio greater than 15, indicating that three types of bedding could continue to be used.

Water content in compost not only serves as a nutrient carrier for microbial metabolism and physiological activities but is closely related to the composting process [36]. Ideally, the WC of the bedding should be maintained between 40% and 60–65% [37]. During the experiment, the WC of all three groups of bedding fluctuated and increased. However, the

WC of all three groups of bedding (FSD, 44.20%; FST, 57.25%; FM, 54.86%; Figure 1e) was eventually in the desired range and could be continued to be used.

Electricity conductivity describes the variation in total dissolved ions, such as NH_4^+, NO_3^-, Cl^-, Na^+, K^+, SO_4^{2-}, during the fermentation process [12,38]. From d 1 to 6, the EC of the FST and FM was higher than that of the FSD ($p < 0.01$), followed by a progressive drop at d 11 (Figure 1f). EC was significantly higher in FSD than in FST at 11–31 d ($p < 0.05$). The decrease in EC in FST and FM might be caused by the decrease in NO_3^--N and the increase in pH, while the increase in EC in FSD was just the opposite. The correlation analysis revealed that the EC processed a positive association with NO_3^--N and a negative relationship with WC and pH ($p < 0.05$; Figure 6).

pH value is an important factor affecting functional microbial communities and available nitrogen content [39]. During the whole period, the pH of the FST, FSD and FM fluctuated from 8.66 to 9.25, 8.46 to 7.89, and 6.76 to 7.92; the pH of the FST was significantly higher than that in the other groups ($p < 0.01$; Figure 1g). The decrease in pH in FSD was due to stronger nitrification, while the increase in pH in FST and FM was due to weaker nitrification. Nitrification produces two moles of H^+ by converting one mole of NH_4^+ to one mole of NO_3^-, resulting in a decrease in pH [40]. Correlation analysis showed that pH was negatively correlated with EC and NO_3^--N ($p < 0.01$; Figure 6). The optimal pH value in the composting process is between 7.5 and 8.5 [41], and the pH values of FSD and FM were conducive to microbial activity in the bedding.

NH_4^+-N and NO_3^--N are the primary forms of nitrogen involved in nitrification and denitrification. Throughout the test period, the NO_3^--N in FST and FM decreased by 61.80% and 65.70%, respectively, while the NO_3^--N in FSD rose by 45.26%. From d 1 to d 26, the concentration of NO_3^--N in FM was higher than that in FSD and FST ($p < 0.001$; Figure 1h). NO_3^--N was significantly higher in FSD than in the remaining two groups at d 31. The NH_4^+-N in all groups varied from the entire experimental period. The NH_4^+-N in FSD and FM rose 8.44% and 129.46%, respectively, while the NH_4^+-N in FST dropped 40.31% on d 31, compared with that on d 1 (Figure 1i). Correlation analyses showed that the pH and WC were negatively correlated with NO_3^--N concentration ($p < 0.05$, Figure 6). A decrease in pH has been reported to promote NO_3^--N formation during composting [42]. Compared with the initial stage, the cumulative increase in NO_3^--N and NH_4^+-N in FSD was 467.05 mg/kg, the decrease in FST was 968.08 mg/kg, and the decrease in FM was 1,112.44 mg/kg, indicating FSD had stronger nitrogen fixation effect on inorganic nitrogen.

3.2. Emissions of CO_2, N_2O, NH_3 and CH_4 in Bedding Materials

In this study, concentrations of gases, including CO_2, N_2O, NH_3 and CH_4, at a depth of 15 cm of the bedding were examined (Figure 2). It is known that CO_2 emission is an important indicator for evaluating microbial activity in composting [35]. From d 1 to d 21, CO_2 concentration fluctuated and increased in FST and FM, with the maximum concentration 45,516.67 mg/m^3 in FST on d 16 and 26,550.33 mg/m^3 in FM on d 21 ($p < 0.001$; Figure 2a). The CO_2 concentration in FSD fluctuated between 2165.03 and 8170.40 mg/m^3 for 1–21 days. From d 26 to 31, the CO_2 concentration in all groups was low, ranging from 809 to 845 mg/m^3.

Oxygen concentration within the compost is an important factor in the production of CH_4, which is produced by methanogenic bacteria under anaerobic conditions [43]. The CH_4 concentrations in the three bedding groups showed a general trend of increasing and then decreasing (Figure 2b). The CH_4 concentration peaked on d 21 in the FSD (145.40 mg/m^3) and on d 16 in FST (247.87 mg/m^3) and FM (169.14 mg/m^3).

N_2O is produced due to incomplete nitrification or denitrification during storage, and denitrification is the main source of N_2O emissions under low oxygen or anaerobic conditions [44]. N_2O concentrations in FST and FM increased and then decreased (Figure 2c). The FSD reached a higher concentration of N_2O on d 6 (34.47 mg/m^3), which fluctuated between 10.5 mg/m^3 and 37.55 mg/m^3 from d 6 to d 21. N_2O concentrations peaked at day 16 in all three bedding groups (FSD, 37.55 mg/m^3; FST, 153.43 mg/m^3; FSD, 71.47 mg/m^3).

NH_3 is produced through the breakdown of nitrogenous substances (i.e., proteins and amino acids). The concentration of NH_3 was significantly higher in FM than in FSD and FST at d 6 and 21 ($p < 0.05$; Figure 2d). This may be related to the higher fermentation temperature in FM, where a large amount of NH_3 escaping from the bedding causes a loss of nitrogen [45].

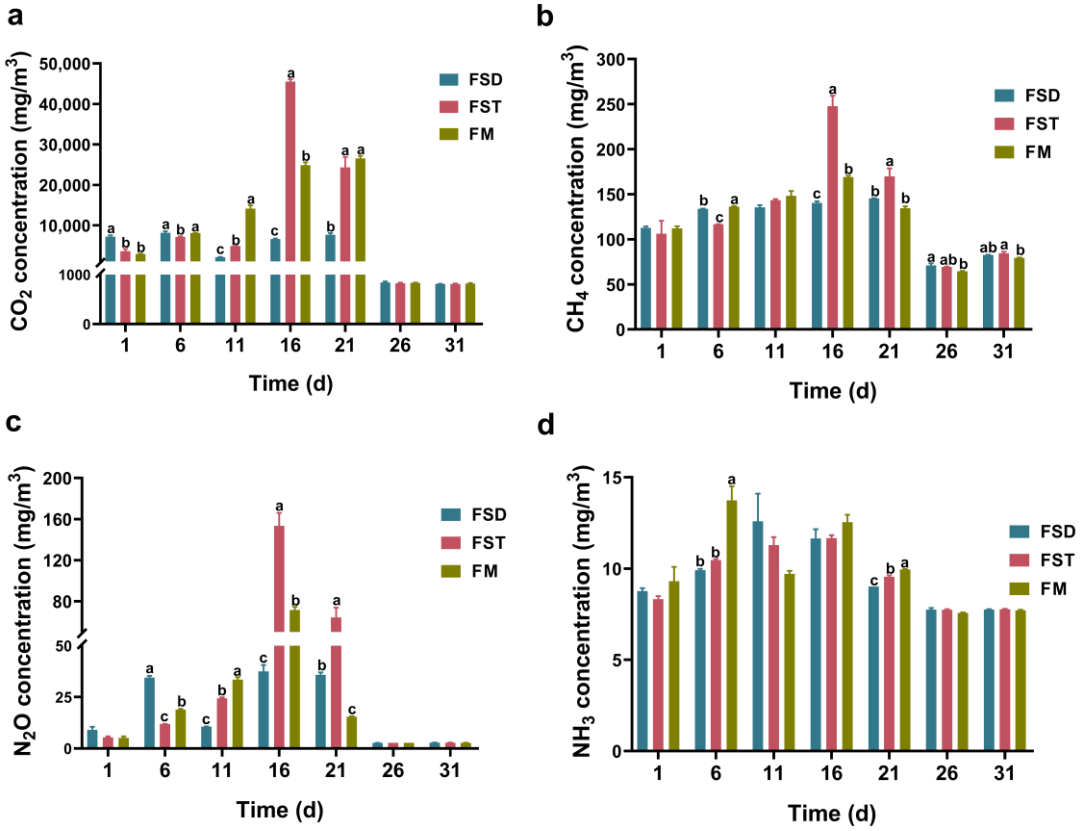

Figure 2. Concentrations of CO_2, CH_4, N_2O and NH_3 in both groups. Changes in CO_2 (**a**), CH_4 (**b**), N_2O (**c**) and NH_3 (**d**) concentrations of three litters during the feeding experiment. Data are means ± SEM, $n = 5$. FSD, fermented sawdust; FST, fermented straw; FM, fermented sawdust–straw mixture. Letters a, b and c show significant differences ($p < 0.05$) between the three groups at the indicated time points.

3.3. Changes in Microbial Diversity and Abundance

With an average of 78,473 (bacterial) and 62,120 (fungal) sequences per sample, 2,118,772 and 1,677,229 high-quality bacterial and fungal sequences were retrieved from all samples. The Chao 1 index is used to evaluate the richness of the microbial community, while the Shannon index is used to reflect the diversity of the microbial community [46]. As shown in Table S1, bacterial Chao 1 and Shannon indexes in the bedding materials of the three groups gradually increased from day 1 to day 16 ($p < 0.05$), and the Shannon index in FST was higher than that in FSD ($p < 0.05$). Chao 1 and Shannon indices of fungi in FST increased gradually from d 1 to d 16. The Shannon index of fungi in FM also increased from d 1 to 16. At d 16, the fungal Chao 1 and Shannon indices in FST were higher than those in FM ($p < 0.05$). This indicates that the microbial community diversity and richness were higher in the FST at d 16.

The shared and unique bacterial and fungal species showed opposite evolutionary trends under the different bedding materials (Figure 3a,b). The number of shared bacterial species of the bedding was three times more than that of unique species, while shared fungal species accounted for only about seventy percent of the unique species. This suggests that the similarity of the bacterial communities of the three bedding groups was high, while

these groups possessed a high level of uniqueness in the fungal communities. The bacterial and fungal community compositions of the two matrices were compared using PCoA. As shown in Figure 3c,d, PCoA1 and PCoA2 explained 47.43% and 12.26% of the total variance of the bacterial community and 18.69% and 15.35% of the total variance of the fungal community, respectively. Treatments and time for the bacterial (treatments: R = 0.237, $p < 0.01$; time: R = 0.709, $p < 0.001$) and fungal communities (treatments: R = 0.329, $p < 0.001$; time: R = 0.354, $p < 0.001$) were significant.

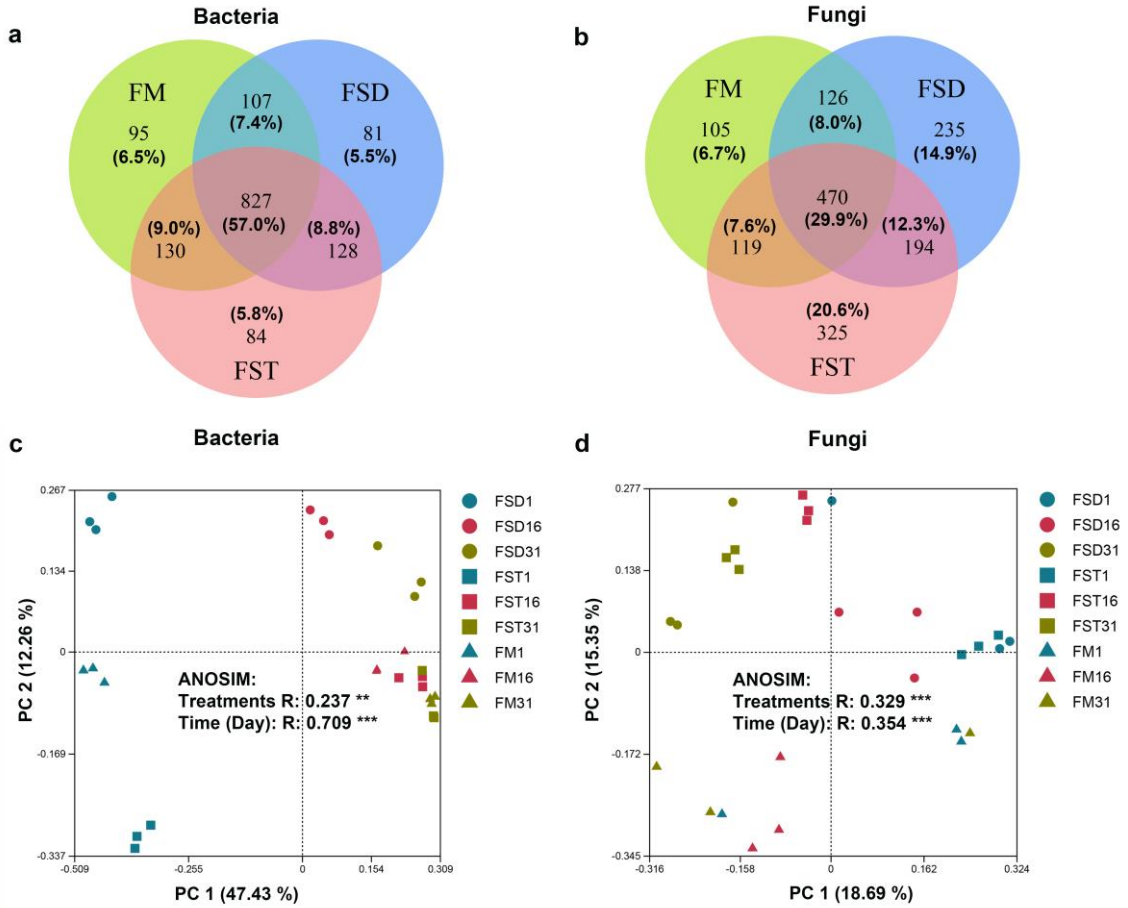

Figure 3. Microbial community composition in three groups. Venn diagram shows shared and unique bacterial (**a**) and fungal (**b**) species, respectively. The PcoA analysis (Bray–Curtis) of bacteria (**c**) and fungi (**d**) based on ASVs. FSD, fermented sawdust; FST, fermented straw; FM, fermented sawdust–straw mixture. ** $p < 0.01$, *** $p < 0.001$.

3.4. Evolution of Microbial Community

Based on 97% sequence similarity, a total of 22 and 10 phyla, 43 and 36 classes, 123 and 82 orders, 239 and 183 families, 437 and 330 genera, 497 and 467 species of bacteria and fungi were observed, respectively, using Illumina novaseq 6000 platform.

The relative abundance of the dominant bacterial phyla composition is shown in Figure 4a. *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Firmicutes*, *Chloroflexi* and *Gemmatimonadota* were the six primary phyla in three groups (Figure 4a). The relative abundance of *Proteobacteria* gradually increased in the three groups (FSD, 23.28–40.35%; FST, 39.80–46.71%; FM, 26.63–50.87%). *Proteobacteria* play an important role in the degradation of compost-containing plant residues [47], and many bacteria with this genus have been functionally identified as organisms associated with the carbon and nitrogen cycle [48]. The LEfSe analysis indicated that *Proteobacteria* were enriched in FM31 (Figure 5a), indicating that the FM has a stronger capacity for carbon and nitrogen turnover. Correlation analysis showed that *Proteobacteria* were positively correlated with TN, NH_4^+-N and WC

and negatively correlated with NO_3^--N, C/N ratio, EC, OM, CH_4, NH_3 and N_2O ($p < 0.05$; Figure 7a). The relative abundance of *Actinobacteria* (FSD, 15.52–9.22%; FST, 23.62–11.60%; FM, 22.94–9.82%), *Chloroflexi* (FSD, 32.46–3.41%; FST, 3.59–1.10%; FM, 13.19–0.60%) and *Gemmatimonadota* (FSD, 3.00–1.07%; FST, 2.82–2.12%; FM, 9.88–2.86%) gradually decreased (Figure 4a). Current evidence suggests that *Chloroflexi* is involved in the second step of nitrification, which is the oxidation of NO_2^- to NO_3^- [49]. *Chloroflexi* was significantly enriched in FSD1 (Figure 5a), and NO_3^--N content in FSD increased by 45.26% from d 1 to d 31, whereas it decreased by 61.80% and 65.70% in FST and FM, respectively. This result suggested that nitrification in FSD was stronger than that in FM and FST, which is favorable for N fixation. Correlation analysis showed that the *Chloroflexi* were positively correlated with the OM, EC, C/N ratio and NO_3^--N and negatively correlated with the WC, NH_4^+-N and TN ($p < 0.05$; Figure 7a). The LEfSe analysis showed that *Actinobacteria* was enriched at FST1 (Figure 5a). *Actinobacteria* can break down stubborn polymers such as lignocellulose and participate in carbon and nitrogen nutrients [50,51]. Correlation analysis showed *Actinobacteria* were negatively correlated with temperature, WC and TN ($p < 0.05$; Figure 7a). The relative abundance of *Bacteroidota* in FSD gradually increased from 9.65% to 26.65%, while that of the remaining two groups first increased and then decreased. Additionally, *Bacteroidota* was enriched at FSD16 (Figure 5a). The relative abundance of *Firmicutes* decreased and then increased in FSD and FST while gradually increasing in FM.

At the bacterial genus level, *Unclassified_SBR1031* (29.71%) was the microorganism with the highest relative abundance in FSD1 (Figure 4b), followed by a gradual decrease in relative abundance (FSD31, 2.78%). Correlation analysis showed that *Unclassified_SBR1031* was positively correlated with EC, NO_3^--N, C/N ratio and OM, and negatively correlated with TN, WC and NH_4^+-N ($p < 0.05$; Figure 7b). *SBR1031* belongs to the *Chloroflexi* and is a nitrifying bacterium that can convert NH_4^+-N into NO_3^--N [52]. The relatively high abundance of *Unclassified_SBR1031* in FSD1 promotes the generation of NO_3^--N. Additionally, several nitrogenous compounds provided satisfactory nutritional conditions for *SBR1031* [53], playing a role in the TN and C/N ratio. The production of NO_3^--N and NH_4^+-N was responsible for the change in EC value [38]. *Ornithinicoccus* (10.45%) was enriched in FM1 (Figure 5a), which was the potential common host of tetracycline resistance genes (tetC, tetG and tetX) and sulfonamide resistance genes (sul1 and intI1) [54].

As shown in Figure 4c, *Ascomycota* and *Basidiomycota* had the highest relative abundance among the three groups of bedding materials at the fungal phylum level, accounting for about 90% of the total sequence. Both *Ascomycota* and *Basidiomycota* play important roles in lignocellulosic degradation [55,56]. The relative abundance of *Ascomycota* decreased gradually in FSD and FST (FSD, 80.78–71.92%; FST, 78.72–74.42%) but increased in FM (73.92–83.85%; Figure 4c). The relative abundance of *Basidiomycota* in the three groups decreased gradually. *Basidiomycota* was significantly enriched in FST31, and *Ascomycota* was significantly enriched in FM1 (Figure 5b). *Crassicarpon* was the most dominant microorganism in all three bedding groups at the genus level. A gradual decrease in the relative abundance of *Crassicarpon* in the FSD and FST was observed (FSD, 21.28–2.33%; FST, 20.95–5.03%), with little change in FM (FM, 17.53–17.19; Figure 4d).

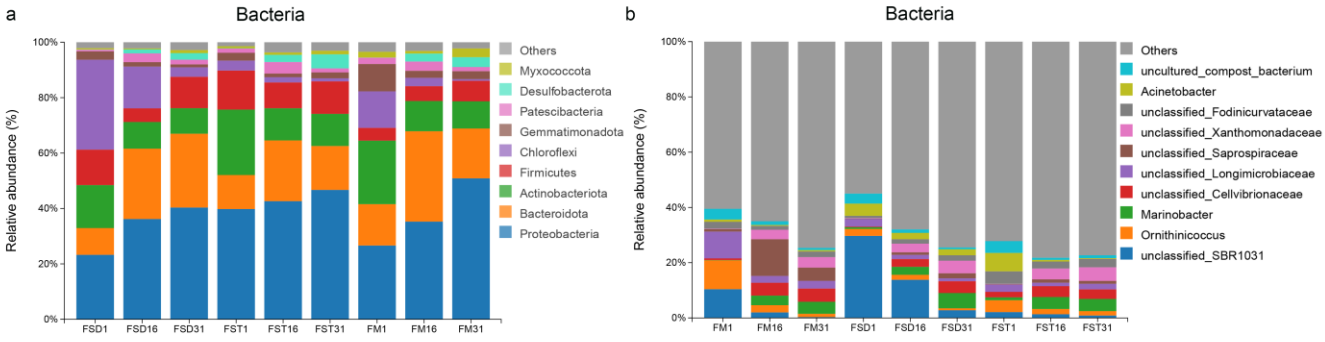

Figure 4. *Cont.*

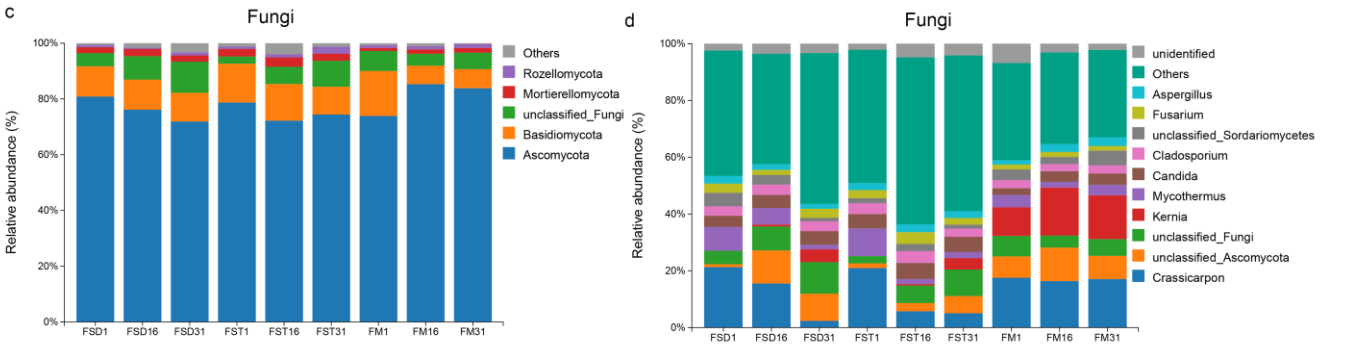

Figure 4. Relative abundance of dominant microbial composition at the phylum and genus levels. The relative abundance of bacterial and fungal phyla (**a,b**) as well as bacterial and fungal genera (**c,d**) in all samples. FSD, fermented sawdust; FST, fermented straw; FM, fermented sawdust–straw mixture. Phyla and genera with a mean relative abundance of more than 1% in at least one group are shown.

Figure 5. LEfSe analysis of bacterial and fungal taxa. Indicators of bacterial (**a**) and fungal (**b**) in groups with LDA scores higher than 4. All the bacterial communities or species displayed have

statistical differences among all groups. The letters p, c, o, f, g and s stand for the abbreviation of kingdom, phylum, class, order, family, genus and species. The bacterial community or species with significant abundance differences in different groups are shown, and the length of the histogram represents the influence of biomarkers. FSD, fermented sawdust; FST, fermented straw; FM, fermented sawdust–straw mixture.

3.5. Influence of Environmental Factors on the Microbial Community

The Mantel tests revealed that bacterial and fungal community composition was mainly driven by the bedding environmental factors (Figure 6). The TN (r = 0.411), C/N ratio (r = 0.546), WC (r = 0.559), NH_4^+-N (r = 0.320), OM (r = 0.284), EC (r = 0.321) and NO_3^--N (r = 0.257) drove bacterial community composition (Table S2). Fungal communities were regulated by TN (r = 0.412), WC (r = 0.338), NO_3^--N (r = 0.237), pH (r = 0.224), C/N ratio (r = 0.221), EC (r = 0.172) and temperature (r = 0.192). The C/N ratio, WC and TN were the physicochemical factors that had the greatest impact on bacterial and fungal communities. Bacteria have a remarkable ability to adapt to harsh conditions (high temperatures or low pH) compared to fungi, i.e., bacteria are insensitive to temperature and pH [57]. The present results also show that temperature and pH only have a significant effect on fungal communities ($p < 0.05$; Figure 6).

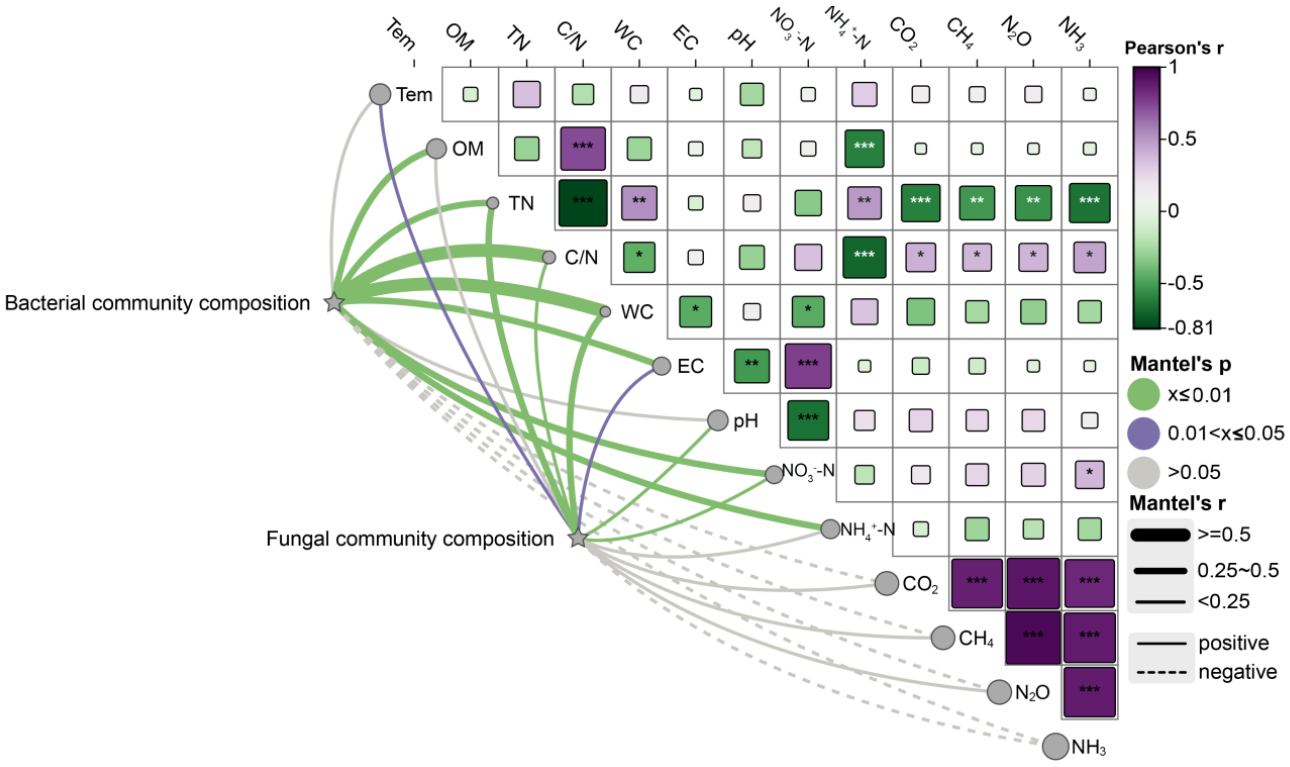

Figure 6. Mantel test showing environmental factors correlations with microbial diversity and communities. Edge width corresponds to the absolute value of the correlation coefficient determined by the Mantel test. Colours indicate the magnitude of the significant correlations. Pairwise comparisons of environmental factors are shown in squares, with colour gradients indicating Pearson's correlation coefficients. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

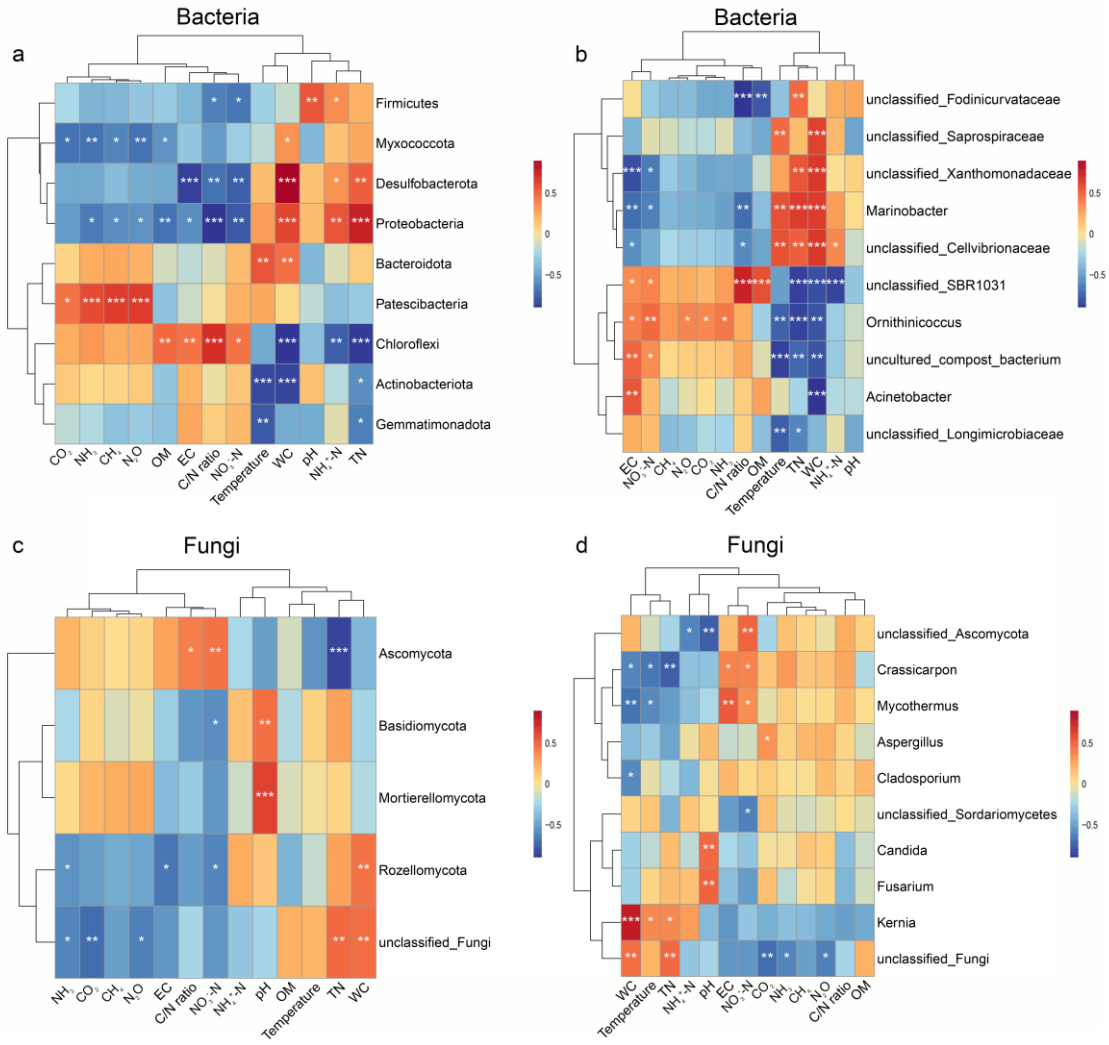

Figure 7. Spearman correlation analysis heat map. Heatmap showing correlations between environmental factors and representative bacterial or fungal phyla and genera based on the Spearman correlation coefficient in the bacterial (**a**,**c**) and fungal (**b**,**d**) community composition. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4. Conclusions

The FSD and FM had a greater capacity to degrade OM, accumulate TN and process a more stable pH environment. Compared with the FST and FM, the relatively lower emissions of N_2O, CH_4 and CO_2 in FSD may be related to its increased carbon sequestration (promoting NO_3^--N and NH_4^+-N accumulation). The FM processed higher NH_3 concentration. All three groups of bedding materials had temperatures below 35 °C and moisture contents below 60% during the feeding period. Different treatments and times had significant effects on bacterial and fungal communities. Linear discriminant analysis effect size showed that FSD1 enriched *Chloroflexi* and FST1 enriched *Actinobacteriota*, while FM31 enriched *Proteobacteria*. Bacterial communities were more sensitive to NH_4^+-N and OM, while fungal communities were more sensitive to temperature and pH. In summary, FSD had potential advantages concerning N conversion and C emission reduction for bedding material applications in cow raising.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14061132/s1, Table S1: Summary of MiSeq sequencing of 16S rRNA/ITS1 genes in composting samples; Table S2: Analysis of the variability between univariate environmental factors and bacterial or fungal communities by using the Mantel test.

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Data Availability Statement: The raw reads of the 16S rRNA gene and ITS gene sequencing obtained by our research are accessible under the Sequence Read Archive of the NCBI with the accession numbers PRJNA1079701 (bacterial sequences) and PRJNA1079715 (fungal sequences).

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