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Effect of Extending High-Temperature Duration on ARG Rebound in a Co-Composting Process for Organic Wastes

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Abstract: High levels of antibiotic resistance genes (ARGs) in compost materials pose a significant threat to the environment and human health. Conventional composting (CC) is widely adopted for waste management. However, mitigating ARG rebound in the late phase remains challenging. This work presents a strategy to extend the high-temperature duration by external heating to achieve rapid composting (RC). An innovative two-stage heating mode (first stage: day 3–6, 55 °C; second stage: day 7–10, 70 °C) was utilized in this study. We aimed to compare the removal and the rebound of ARGs and mobile genetic elements (MGEs) between RC and CC treatments and to identify the key factors driving the fate of ARGs throughout the composting process by integrating with environmental factors, external stress, MGEs, and microbial communities. The results show that on day 40, ARGs increased by 8.2 times in conventional composting. After the high-temperature duration was prolonged from 5 days to 9 days, the highest elimination rates achieved for ARGs and MGEs were 85% and 97%, respectively; concurrently, ARG rebound was suppressed compared to conventional composting. Genes resisting β-lactamase, chloramphenicol, and quinolone showed maximal removal in both treatments. The antibiotics showed a significant reduction in both treatments, with 79.3% in extended high-temperature duration composting and 75.26% in conventional composting. Network analysis revealed that *Gammaproteobacteria*, *Clostridia*, *Saccharimonadia*, *Cyanobacteriia*, and *Campylobacteria* were the potential hosts of various ARG subtypes, and their abundance was reduced in extended high-temperature duration composting. Redundancy analysis (RDA) and structural equation model (SEM) confirmed that temperature was the key factor in composting, while the potential hosts of MGEs and ARGs were responsible for the rebounding of ARGs in conventional composting. Prolonging composting temperature is a key strategy for the removal of contaminants from aerobic composting to achieve a safe end-product.

Keywords: aerobic composting; ARG rebound; MGEs; host bacteria; temperature control

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1. Introduction

Antibiotic resistance genes (ARGs), an emerging pollutant, have attracted much attention in recent years due to their widespread distribution and strong dissemination ability [1]. Various ARGs are prevalent in animal manure and food waste because of the overuse of antibiotics [2] The absolute abundance (AA) of ARGs in animal manure has been reported to reach up to 10^{11}–10^{13} copies/g dry weight [3], while in food waste it ranges from 1.88×10^5 to 1.5×10^{10} copies/g dry weight [4]. ARGs can be transmitted among various bacteria, including human pathogens, through horizontal gene transfer facilitated by mobile genetic elements (MGEs) [5]. Consequently, antibiotic treatments may become ineffective once ARGs enter human bodies via human pathogens, thereby posing a threat to global public health [6]. Numerous studies have reported that the application of untreated or ineffectively treated ARG-rich substances can result in the dissemination of ARGs in the environment [7,8]. Hence, it is imperative to appropriately treat these substances to mitigate the risk of human exposure.

Composting is an eco-friendly technique for organic solid waste disposal; previous studies have confirmed its positive effect on ARG removal [4,9]. For instance, Qiu et al. [10] adopted conventional composting (CC) to treat chicken manure on an industrial scale and observed a reduction in most ARGs following composting. Similarly, Gao et al. [11] achieved complete elimination of *mcr-1* from cattle manure through aerobic fermentation. However, recent studies have also identified that CC may not completely eradicate all identified ARGs from organic waste [7,12]. Specifically, the prolonged operation cycle and the widespread rebounding phenomenon of ARGs in the cooling period are the primary obstacles [13]. Subirats et al. [14] noted an enrichment in the relative abundance (RA) of selected ARGs (*ermF*, *sul1*, *strA*, *strB*, $bla_{OXA20,}$ and *blaPSE*) in the final compost. A similar result was also reported by another study, which showed that the total RA of ARGs increased by 1.7–4.9-fold during the cooling phase of composting [13]. Nevertheless, the fate of ARGs in composting remains unclear. Unlike the natural environment, composting systems are extremely complex due to changes in the environmental conditions, rapid shifts in microbial composition, and substantial variations in external stress [1,15], making the mechanism of the degradation, residue, and rebounding of ARGs unclear.

Previous studies have reported different driving factors in various composting systems. For instance, microbial communities and MGEs have been confirmed to promote the spread and amplification of ARGs through vertical and horizontal gene transfer. Heavy metals and antibiotics sustainably induce ARGs through selection pressure, while the environmental conditions mostly affect ARGs indirectly by influencing microorganisms or external stress [1,15]. Liao et al. [16] reported that MGEs shaped the fate of ARGs in hyperthermophilic sludge compost, while the microbial community contributed the most in conventional sludge composting. Moreover, Yue et al. [17] concluded that antibiotic degradation dominates the removal of ARGs during composting. Liao et al. [18] found that horizontal transfer between microbial communities was the reason for the persistence of ARGs during food waste composting. Furthermore, the incomplete degradation of DNA containing ARGs and/or MGEs and high-temperature-tolerant host bacteria are widely believed to be responsible for ARGs rebounding at the cooling stage [15,16,19]. However, the evolution mechanism of ARGs in the composting process remains largely unrevealed, meaning a comprehensive analysis based on multi-parameters is urgently needed.

Temperature is a key controlling factor for both improving composting efficiency and enhancing ARG removal [16,20]. According to Selvam et al. [21] and Liao et al. [16], maintaining the temperature between 55 °C and 65 °C was essential for efficient and rapid composting and hyperthermophilic conditions were conducive to reducing and preventing ARGs rebounding. Qian et al. [22] compared the effects of three different whole-process heating modes on ARG removal during composting and found that both normal thermophilic composting and continuous thermophilic composting achieved greater decreases in selected ARGs and two integrons than insufficient thermophilic composting. There are varied opinions in the literature regarding the effect of composting temperature on ARGs. For example, Wu et al. [23] recommended thermophilic digestion for attenuating ARGs and MGEs, while Huang et al. [24] noted that elevated temperatures might not always ensure complete removal of ARGs and MGEs. Numerous studies have reported on the effects of temperature on ARG removal; however, the reasons for enhanced removal by temperature control are rarely discussed.

This study presents a strategy to extend the high-temperature duration by external heating to achieve rapid composting (RC). An innovative two-stage heating mode (first stage: day 3–6, 55 °C; second stage: day 7–10, 70 °C) was utilized in this study. We aimed to compare the removal and the rebound of ARGs and MGEs between RC and CC treatments and to identify the key factors driving the fate of ARGs throughout the composting process by integrating with the environmental factors, external stress, MGEs, and microbial communities. This study aims to provide insights into the fate of ARGs in the composting process and to offer a promising strategy for controlling the rebounding of ARGs.

2. Methods and Materials

2.1. Composting Materials and Experimental Setup

The details about the composting materials are presented in our previous works [12,25]. In brief, the composting materials included swine manure, food waste, and cornstalk that were collected from a swine farm, the campus canteen, and an agricultural processing plant in Shanghai, respectively. The raw materials were thoroughly mixed with a wet mass ratio of 2:2:1 (swine manure: food waste: cornstalk). The initial moisture content was adjusted to approximately 65% with a ratio of C to N of 27:1.

The co-composting experiments were conducted in lab-scale reactors (25 L) for a period of 40 days. Two composting processes were performed simultaneously in three repetitions, which were conventional composting (CC) and rapid composting (RC) with temperature control. The RC process employed a two-stage heating mode: the first stage, spanning from day 3 to day 6, maintained a temperature of 55 °C, followed by the second stage, lasting from day 7 to day 10, with the temperature raised to 70 °C. The temperature was regulated by the heating belt wrapped around the reactors. In contrast, conventional composting had the same conditions as rapid composting but without any external heating. Adequate aeration was ensured by manually turning the composting material once every two days to maintain the aerobic conditions and even heat distribution. Deionized water was added during the mesophilic and thermophilic stages to keep the moisture content at 60%.

2.2. Sampling and Physicochemical Analysis

Samples (50 g) were collected from each reactor on days 0, 1, 2, 3, 5, 7, 10, 14, 30, and 40. The samples were thoroughly mixed and divided into two parts and stored at −20 °C for physicochemical analysis and −80 °C for biological analysis.

Physicochemical analyses are described in detail in our previous works [12,25]. In brief, the core temperature was measured twice a day using a digital thermometer. The pH of the compost was determined by measuring the extract of the compost samples with a pH meter. The germination index (GI) was measured according to organic fertilizer (NY/T 525-2021 [26]). The organic matter (OM) and the total organic carbon (TOC) were quantified using the burning method. Kjeldahl nitrogen was determined with Kjeldahl apparatus (Lichen, China).

2.3. Determination of Heavy Metals and Antibiotics

To extract heavy metals, the freeze-dried sample was added to concentrated nitric acid for digestion. The initial temperature of the heating plate was set at 120 °C for 30 min and then increased to 180–190 °C for 1–2 h. The digestion products were filled to 25 mL with ultra-pure water and stored at 4 °C after filtration. The different speciation of heavy metals was extracted using an improved BCR method [27]. The extracted heavy metals were quantified using an inductively coupled plasma mass spectrometer (ICP-MS, NexION2000-Flexar20 HPLC, PerkinElmer, Waltham, MA, USA).

Antibiotics were extracted and quantified by the following steps: sample pretreatment, solid phase extraction (SPE), and ultrahigh-performance liquid chromatography tandem mass spectrometry (UPLC-MS-MS) [28]. The targeted antibiotics consisted of 29 antibiotics belonging to four families (macrolides, tetracyclines, sulfonamides, and fluoroquinolones).

2.4. DNA Extraction, High-Throughput Sequencing, and Bioinformatics

The DNA was isolated from freeze-dried samples using the Rapid DNA Spin Kit for Soil (MP Company, Las Vegas, NV, USA), referring to the manufacturer's instructions. The DNA content and the quality were checked with a NanoDrop one ultra-micro spectrophotometer. The extracted DNA was amplified by PCR using a 338F_806R universal primer. The F-end primer was ACTCCTACGGGAGGCAGCAG, and the R-end primer was GGACTACHVGG GTWTCTAAT. The Illumina PE300 sequencing platform was used to perform high-throughput sequencing on PCR products at Shanghai Majorbio Technology Co., Ltd.

The sequences were clustered into operational taxonomic units (OTUs) at a similarity level of 97%.

2.5. High-Throughput Sequencing and Real-Time PCR for Determination of ARG and MGE Abundances

PCR amplification was performed on a Smart Chip Real-Time PCR Systems (WaferGen, Fremont, CA, USA) high-throughput fluorescence quantitative reaction platform. A total of 373 genes were designed, including 360 ARGs, 12 MGEs, and 16S rRNA genes. The quantitative system requires a 100 µL solution containing 1 × Light Cycler 480 SYBR® Green I Master Mix (Roche, Basel, Switzerland), 5 ng/µL DNA, 1 µg/µL BSA, 1 µmol/L primers, and Nuclease-free PCR-Grade water.

The qPCR conditions comprised an initial hold for 10 min at 95 °C, followed by denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and a total of 40 cycles. The program automatically heated up to analyze the melting curve. The amplification efficiency was between 1.8 and 2.2. According to the detection limit and sensitivity of the Smart Chip Real-time PCR System, the detection cycle threshold (C_T) was 31.

A Roche 480 quantitative PCR instrument (Roche company) was used to evaluate the 16S rRNA genes in samples, and the absolute abundance was determined using the standard plasmid external standard method. The reaction volume of the real-time quantitative PCR was 20 µL, including 10 µL 2 × Light Cycler 480 SYBR® Green I Master Mix, 2 µL upstream and downstream primers (10 µmol/L), 1 µL sample or DNA template, and 7 µL Nuclease-free PCR- Grade water.

The absolute quantitative PCR reaction operation procedure was as follows: predenaturation at 95 °C for 30 s, annealing extension at 60 °C for 60 s, extension at 72 °C for 30 s, and a total of 40 cycles. Each sample was assayed in duplicate, and Nuclease-free PCR-Grade water was used as a negative control.

2.6. Statistical Analysis

Excel 2019 was used to calculate and analyze the basic experimental data, which was visualized using Origin 2022b. Origin 2022b was also used to obtain the heatmap of ARGs and MGEs. Species diversity analysis was realized on the online platform of the Majorbio Cloud Platform (http://www.majorbio.com, accessed on 8 May 2022) [29]. The correlation analysis was carried out in SPSS 26.0. R was used for the principal co-ordinates analysis (PCoA), redundancy analysis (RDA), and network analysis. Network analysis was based on the significant pairwise Spearman correlation coefficients ($R > 0.6$, $p < 0.01$). A structural equation model (SEM) was constructed to assess the direct or indirect effects of all potential factors on the patterns of ARGs using AMOS 24.0 software (SPSS, Chicago, IL, USA).

3. Results and Discussion

3.1. Changes in Environmental Parameters

Environmental parameters were monitored during the composting process; the variations are depicted in Figure 1. The temperature profiles of the two composting processes showed different trends. Rapid composting exhibited an extended mesophilic and thermophilic period (Figure 1a). After 5 days of fermentation, the temperature in CC increased to its maximum of 67.5 °C and was maintained at the thermophilic phase (above 50 °C) for 5 days. In contrast, RC reached its highest temperature (70.5) on day 7 and the thermophilic period lasted for 9 days, including 4 days in a hyperthermophilic state that was suitable for the removal of pathogenic bacteria from animal waste. The GI values were analyzed to observe the phytotoxicity of the CC and RC treatments (Figure 1b). The GI values were below the standard during the 10 days of the process and remained stable above 70% by day 14 (80.54%) in CC and by day 9 (93.28%) in RC, illustrating that the maturity time of RC was earlier compared to CC. The pH distribution changed dramatically over time (Figure 1c). The pH of both processes dropped sharply from the initial value of 7.52 during the mesophilic periods due to the hydrolysis of organic matter. With the release of ammonia,

the pH increased to 8.83 (RC) and 9.05 (CC) on day 14. The C/N ratio declined throughout the composting period (Figure 1d). In the RC process, the downward tendency of the C/N ratio terminated on day 14 with a value of 19.04. In the CC process, C/N continued to decline until day 40, reaching 19.29. After 14 days of fermentation, the C/N remained constant in RC, indicating that the compost was stable.

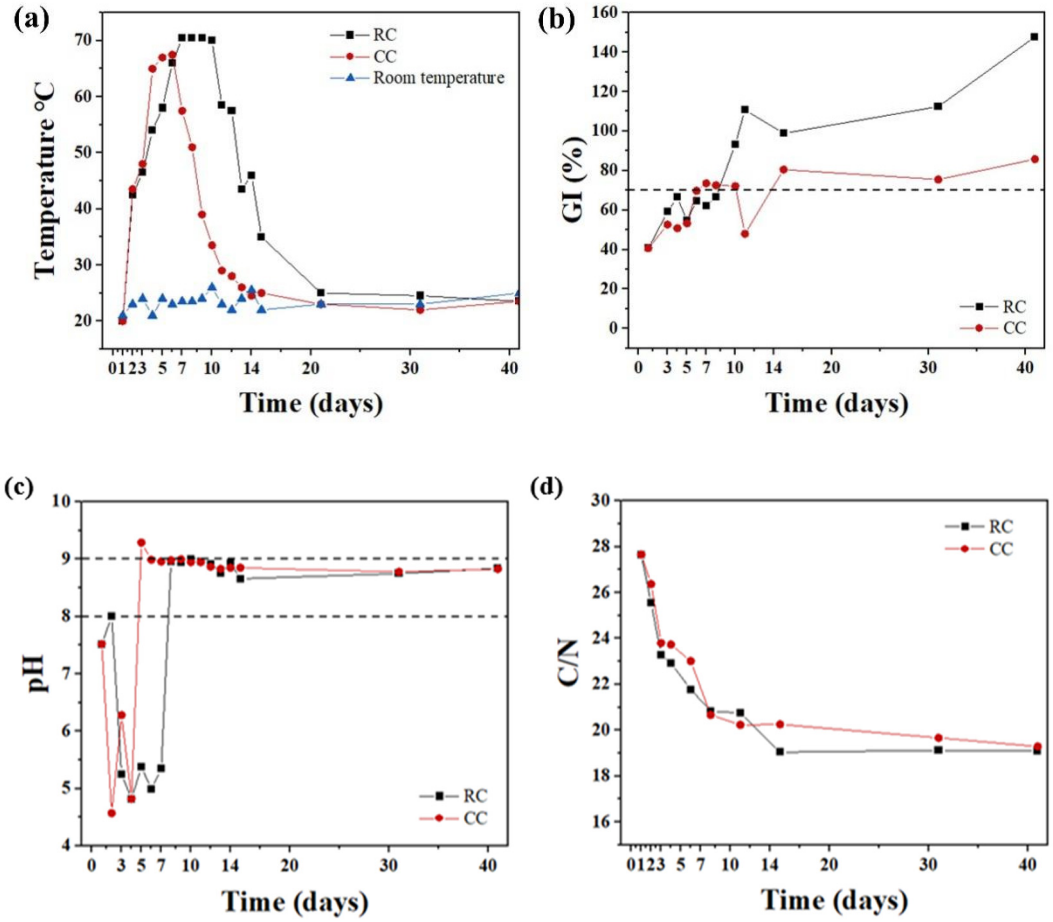

Figure 1. Physicochemical indices variation during composting. (**a**): Temperature; (**b**): GI (germination index); (**c**) pH; and (**d**) C:N ratio variation during composting. CC: conventional composting; RC: rapid composting.

3.2. Antibiotic and Heavy Metal Concentrations in the Composting System

Antibiotics and heavy metals can exert selective pressure on the fate of ARGs. Therefore, the residual concentrations of antibiotics and heavy metals during the composting process were analyzed. A total of 29 targeted antibiotics were detected, belonging to the tetracycline, quinolone, sulfonamide, and macrolide families. These classes of antibiotics have been mostly studied during animal waste treatment, indicating their extensive usage in livestock for various purposes. The initial contents of all antibiotics from high to low concentration were tetracyclines (94%), quinolones (3.4%), sulfonamides (2.5%), and macrolides (0.1%). The total antibiotic content in the initial mixture was 1.91 mg/kg, which was degraded rapidly during the mesophilic and thermophilic periods (Figure 2a). RC treatment exhibited a higher degradation efficiency and a greater removal proportion. The elevated temperature primarily contributed to the highest removal of antibiotics by enhancing the degradation of organic matter. Additionally, it influenced the fate of antibiotic molecules by altering their chemical states and their interaction with the organo-mineral matrix [30]. The final concentrations of antibiotics in RC and CC were 0.39 mg/kg and 0.47 mg/kg and the corresponding removal proportions were 79.36% and 75.26%, respectively. Sulfonamides

(81.069%) and tetracyclines (79.991%) showed the highest removal in RC. RC demonstrated a stronger ability to remove tetracycline antibiotics at all stages. However, fluoroquinolones were persistent in the composting system, consistent with previous reports [30]. Similarly, macrolides exhibited high residual levels after the composting process. Six kinds of heavy metals were detected in the composting samples, from high to low abundance they were Zn, Cu, Cr, Pb, Cd, and As. The concentration of total heavy metals increased from 170.52 to 216.51 mg/kg (26.94%) in RC and from 170.52 to 217.72 mg/kg (27.97%) in the CC process after 40 days of composting (Figure 2b). Similar findings were also reported previously, where the total content of heavy metals was enriched by 10–30% after composting due to weight loss resulting from the degradation of organic wastes [31].

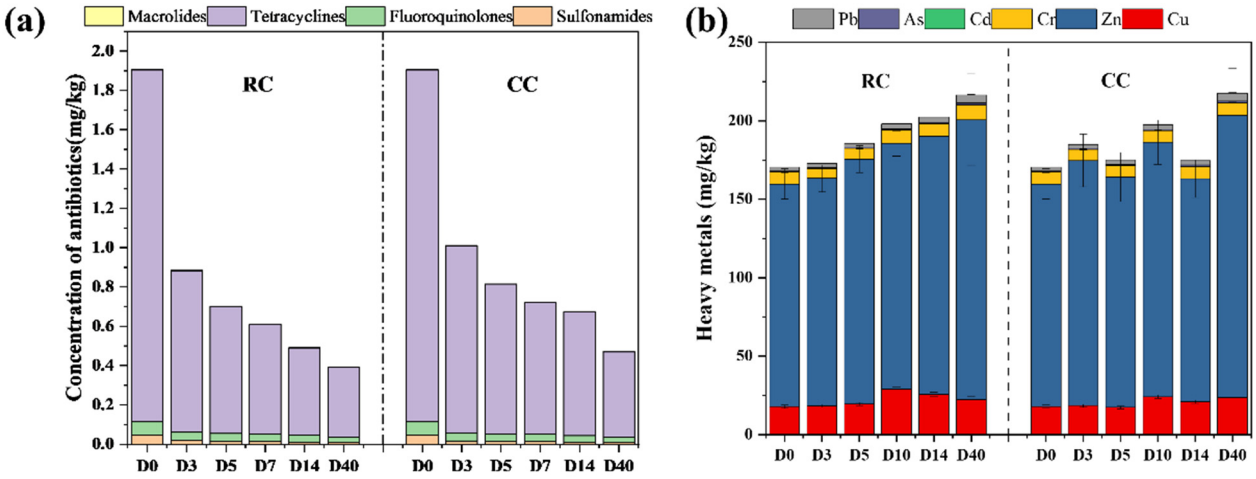

Figure 2. Variation of antibiotics and heavy metals in both composting processes. (**a**) The concentration of antibiotics in both composting processes. (**b**) The variation of heavy metals in both composting processes.

3.3. Fate of ARGs and MGEs in the CC and RC Processes

The variation of ARGs and MGEs during the composting process in both treatments is shown in Figure 3. We targeted 35 ARG subtypes resisting antibiotics, tetracycline, sulfonamide, chloramphenicol, macrolide lincosamide and streptogramin (MLS), quinolones, aminoglycoside, and β-lactamase. Among the targeted genes besides the *mcr-1* and *qnrA* genes, 33 ARG subtypes were detected across the samples. Five MGEs were targeted, including *intI1*, *intI2*, *Tn916/1545*, *ISCR1*, and *tnpA-1.* These MGEs have been frequently reported in the composting process [32,33], but *Tn916/1545* was not detected in this study. The mean concentration of ARGs in the initial mixture was 1.19×10^9 copies/g dry weight. Chloramphenicol and tetracycline resistance genes were the most dominant ARGs initially, accounting for 70.18% of the total, which might be related to the overuse of antibiotics in pig farms [34]. The initial absolute abundance of MGEs was 4.05×10^8 copies/g dry weight, and the MGE subtype *tpnA-1* was predominant at >70% of the total, followed by *intI1* and *intI2*.

During composting processes, the abundance of targeted ARGs and MGEs depicted similar variation patterns in both treatments. With the enrichment in temperature, the abundance of ARGs and MGEs in both treatments declined. On day 10, RC treatments showed the maximal removal of MGEs (6.62×10^7 copies/g dry weight (94.4%)) and ARGs (1.30×10^7 copies/g dry weight (96.8%)). Genes resisting β-lactamase, chloramphenicol, and quinolone showed the maximal removal in both treatments, with β-lactamase being 100% removed in RC treatments. Furthermore, the ARG subtypes *tetQ*, *tetB*, *tetG*, *tetO*, *tetC*, *sul2*, *cfr*, *ermT*, *ermB*, *lnuA*, *mefA*, *ermF*, *ermA*, *qnrS*, *qnrD*, and *oqxA* were completely removed during the thermophilic period. However, the ARGs rebounded during the cooling and maturation phases; similar findings have been reported previously, where ARGs showed a maximum reduction during the thermophilic phase and a resurgence

thereafter [1,16]. ARGs and MGEs rebounded in CC from day 7 onward and gradually recovered to 5.05×10^8 and 5.79×10^7 copies/g dry weight by day 40. The final removal efficiency of ARGs and MGEs on day 40 in CC were 60% and 85% and in RC were 85% and 97%, respectively, which is higher than those reported previously. During the effect of various temperature adjustments on the removal of ARGs, it was observed that the total abundances of the ARGs generally decreased; the average removal efficiencies in the medium (58 °C) and high-temperature treatments (68 °C) were 32.9–34.0%, while numerous ARG subtypes, such as *erm(F)*, were enriched by 2–5 times on day 42 [23]. The minimal removal of ARGs in CC can be attributed to the limited elimination of antibiotics, which likely imposed selective pressure on ARGs, encouraging their retention and resurgence in the later stages. Moreover, the enriched temperature might have eliminated the initial host of ARGs in RC, as it has been previously reported that initial adjustment in composting temperature leads to the maximum removal of ARGs via regulating the initial microbial composition [12]. Moreover, the similar variation patterns for MGEs and ARGs indicate that the fate of ARGs was inextricably linked to MGEs. Several MGE subtypes revived during the cooling period may lead to a lower rebound in ARGs in the RC process. Our results indicate that optimizing the composting temperature can achieve the maximum reduction of ARGs and MGEs while also preventing ARG rebound in the later phases.

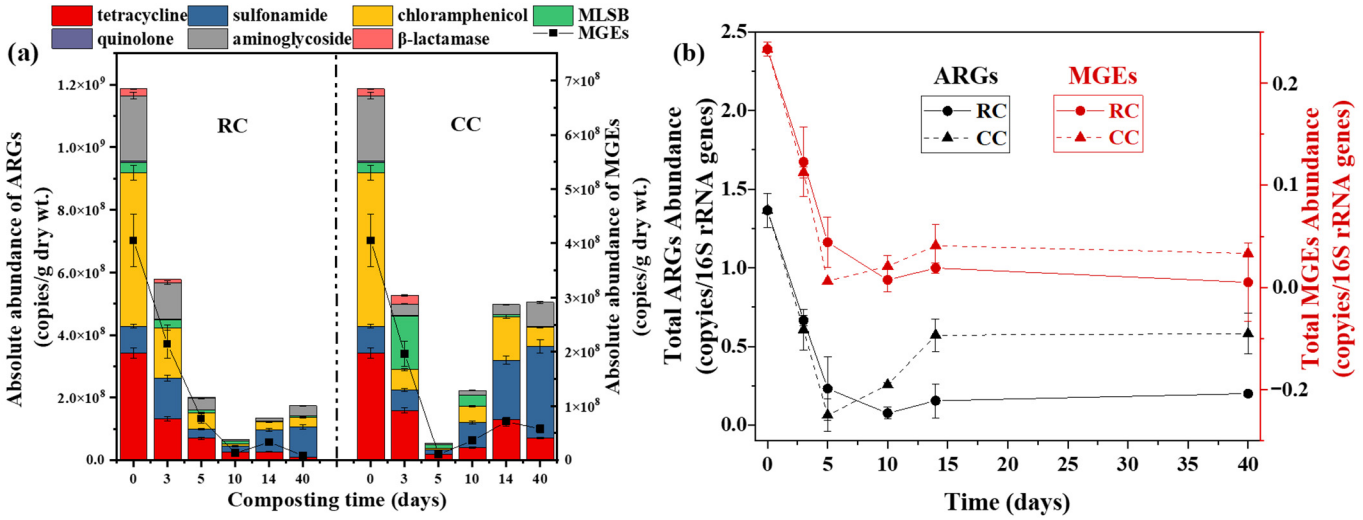

Figure 3. Variation of mobile genetic elements (MGEs) and antibiotic resistance genes (ARGs) during composting. (**a**) Absolut abundance of 7 families of ARGs (left Y-axis) and MGEs (right Y-axis) during both composting processes. (**b**) The relative abundance of total ARGs (left Y-axis) and MGEs (right Y-axis) in the two composting treatments.

3.4. Composition and Succession of Microbial Communities

Biological factors are regarded as the most direct factors influencing the fate of ARGs [1]; thus, the composition and succession of the microbial community were further analyzed. The external heating treatments led to distinct bacterial communities from day 3 according to PCoA (principal coordinates analysis) based on Bray–Curtis distances (at the phylum level) (Figure 4a). The α diversity (Figure 4b) showed the lowest values during the thermophilic period, which is a common evolution tendency in compost [35]. Further analysis of the microbial succession during both composting processes revealed that all sequences were classified into 34 phyla and that the predominant phyla in both treatments were Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidota, accounting for a proportion ranging from 78.0% to 99.5% (Figure 4c). Most Proteobacteria and Bacteroidota are mesophiles and therefore remained dominant in the initial phases. Therefore, considering the close relationship between Proteobacteria and the decomposition of small-molecule organic matter and mineralization of nitrogenous organic [32,36], the strategy of prolonging

the mesophilic period was necessary for the full degradation of organic matter. On the contrary, Firmicutes are heat tolerant [37] and remained dominant in the hyperthermophilic stage. During the cooling stage, the proportion of Actinobacteria gradually increased, which could degrade cellulose and lignin [38]. Wang et al. [39] also proposed that the relative abundance of Actinobacteria in the later phases is an indicator of compost maturity.

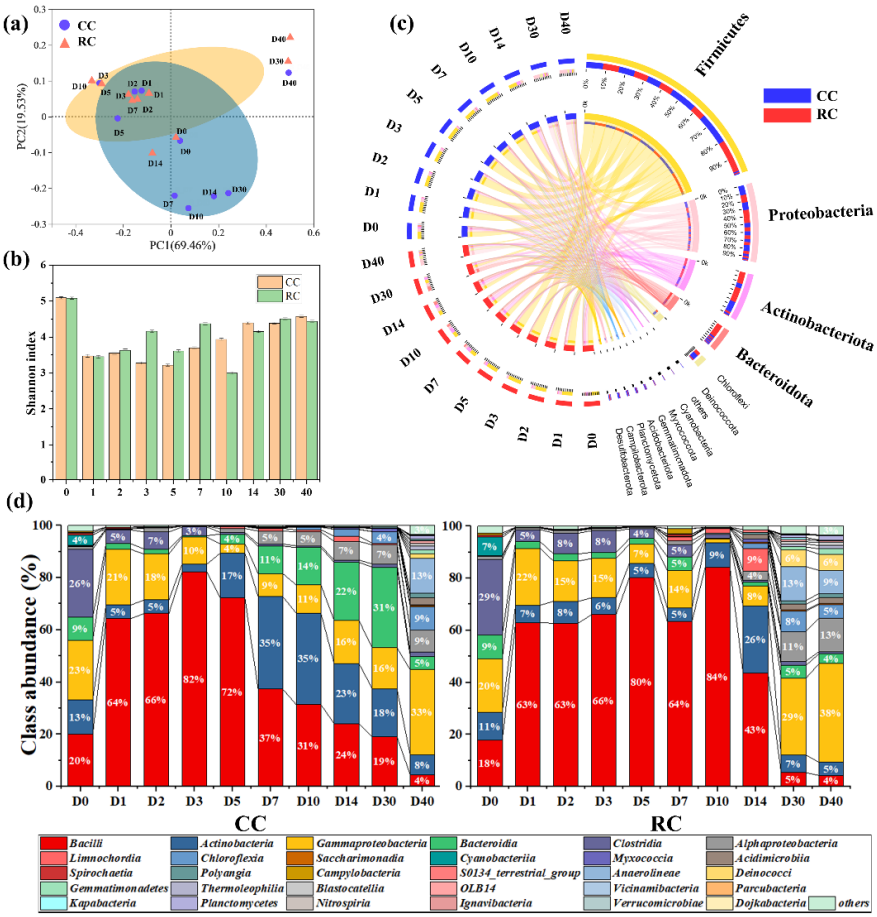

Figure 4. Composition and succession of microbial communities in both composting processes. (**a**) PCoA (principal co-ordinates analysis) based on Bray–Curtis distances (at phylum level); (**b**) alpha diversity; (**c**) distribution of the microbial community for RC and CC at the phylum level. The data were visualized using Circos. The widths of the bars for each phylum indicate the relative abundance of that phylum in the sample. (**d**) Succession of microbial communities during composting (on class level).

At the class level, the microbial composition was significantly different in the RC and CC treatments (Figure 4d). In the initial piles, the predominant classes were *Bacilli*, *Actinobacteria*, *Gammaproteobacteria*, *Bacterodia*, *Clostridia*, and *Cyanobacteria*, accounting for 90% of the entire composition. *Bacilli*, which belong to Firmicutes and are heat-resistant bacteria [40], were dominant as the composting temperature increased, rising from 20% to 80% by day 3 in CC and by day 5 in RC. Bacilli remained the dominant class in RC until day 14, accounting for 43% of the microbial community. Correspondingly, *Actinobacteria*, *Limnochordia*, *Alphaproteobacteria*, and *Gammaproteobacteria* gradually occupied more ecological niches from day 14, with a total relative abundance of 47% in RC. Moreover, with the decrease in temperature in CC, the proportion of *Bacilli* gradually decreased, while *Actinobacteria*, *Gammaproteobacteria*, and *Bacteroidia* became the dominant classes on day 7, with a total proportion of 55%. On day 40, a higher bacterial community diversity was observed in the RC process due to the regrowth of certain bacterial species. These results indicated that RC and CC had different microbial compositions and succession, which

may be one of the explanations for the difference in the changes of ARGs between the two composting processes.

3.5. Potential Host Contributed to the Rebounding of ARGS

The non-random co-occurrence pattern of biotic factors in both processes was further analyzed by network analysis (Figure 5a,b), which can reveal the potential host of ARGs in compost. The co-occurrence pattern of ARGs and MGEs and potential host bacteria in CC was divided into three modules, accounting for 42%, 38%, and 20%. Microbes in the same module with ARGs and MEGs might be the potential hosts and ARGs and MGEs may even share the same hosts. *Bacilli* showed a correlation with *ermA* and *tetC* ($p < 0.05$). *Gammaproteobacteria* was significantly associated with 12 ARG subtypes (*tetA*, *tetM*, *dfrA1*, *cmlA1*, *cmlA5*, *cfr*, *floR*, *ermX*, *mefA*, *qnrS*, *aac(6′)-Ib*, and *strB*) and three MGE subtypes (*intl1*, *intl2*, and *tpnA-1*). Similarly, *Clostridia*, *Saccharimonadia*, *Cyanobacteriia*, and *Campylobacteria* might be the potential hosts of various ARG subtypes, showing associations with *tetA*, *tetB*, *tetO*, *tetM*, *tetW*, *tetQ*, *dfrA1*, *cmlA1*, *cmlA5*, *cfr*, *ermX*, *ermT*, *mefA*, *qnrS*, *aac(6′)-Ib*, and *strB*. Significant positive correlations were also observed for *Spirochaetia* and *tetB*, *tetM*, *tetW*, *tetQ*, *ermT*, *mefA*, and *qnrS* ($p < 0.01$). Figure 5a shows the co-occurrence pattern of ARGs and MGEs and potential host bacteria in RC, which was divided into three modules. Modules 1 to 3 accounted for 39.58%, 37.5%, and 22.92%. A strong relationship ($p < 0.05$) between five ARG subtypes (*tetA*, *tetC*, *cmlA5*, *ermT*, and *sul2*) and *Clostridia* was observed, while *Limnochordia*, *Myxococcia*, *Polyangia*, *Cyanobacteriia*, *Spirochaetia*, and *Acidimicrobiia* were found to be significantly associated with multiple ARGs (*strA*, *ermA*, *tetW*, *ermX*, *floR*, *blaTEM*, *tetB*, *strB*, *aac (6′)-Ib*, *aadB*, and *dfrA1*) and one MGE (*tpnA-1*). Interestingly, 11ARG subtypes, including *ermQ*, *mefA*, *ermB*, *tetQ*, *tetM*, *tetO*, *lnuA*, *qnrD*, *qnrS*, *tetX*, and *cfr*, and two integrons, *intI1* and *intI2*, were associated with the bacteria *Chloroflexia* and *Actinobacteria* ($p < 0.05$), respectively.

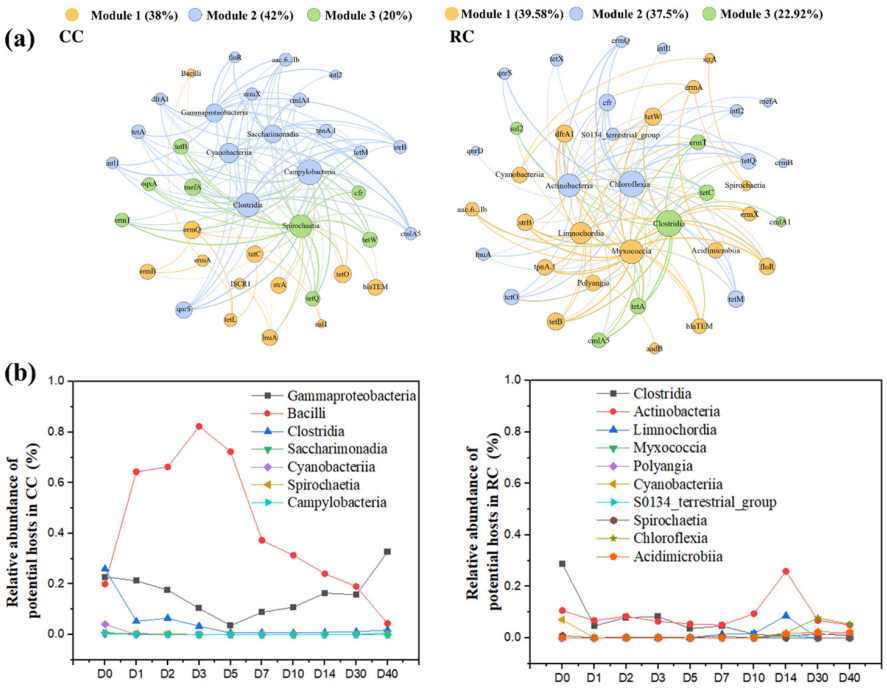

Figure 5. Potential hosts of ARGs and relative abundance of potential hosts. (**a**) Network analysis of the co-occurrence of ARGs, bacterial communities, and MGEs in compost. Different colors represent different related modules; all genes in the same module connected by lines have strong co-occurrence, the thickness of the lines represents the strength of the correlation and the size of the node circle represents the number of genes associated with it (the more, the bigger). The network exhibited significant positive correlations at $p < 0.05$. (**b**) Relative abundance of potential hosts in compost.

Actinobacteria, Firmicutes, Proteobacteria, and Chloroflexi were found to be the main hosts of ARGs in this study, which has also been widely reported in previous studies [41,42]. It was found that Proteobacteria and Firmicutes were the most dominant bacteria carrying tetracycline resistance genes [41]. The *tetC*, *tetX*, *sul2*, and *dfrA7* were possibly affiliated with Chloroflexi during cow manure composting [43]. In addition, the results also indicated that some bacteria, such as *Cyanobacteria*, *Spirochaetota*, and *Campilobacterota*, were not predominant in compost and might have also harbored ARGs. Further analysis of the changes in the relative abundance of potential host microbes (Figure 5b) showed that the rebounding of ARGs depended on the ecological niche occupied by the host during the cooling and maturation phase. The relative abundance of each potential host in RC did not exceed 30% and the slight rebound after day 10 was mainly caused by *Actinobacteria*. There were only seven possible hosts in CC, where *Bacilli* and *Gammaproteobacteria* predominated in different stages of composting. Particularly, *Bacilli* might have protected ARGs with a heat-resistant advantage and the relative abundance of *Gammaproteobacteria* increased from day 5 onward, which was assumed to be the prime host of multiple ARGs in the later phases.

To sum up, the non-random co-occurrence of potential bacteria, MGEs, and ARGs was frequently observed during composting. In RC, the enrichment of potential hosts did not occur during the cooling period, thereby inhibiting ARG rebounding. Therefore, by selectively eliminating key potential hosts through temperature control, the resurgence of ARGs in the later stages of composting can be mitigated.

3.6. In Situ Drivers of ARGs in the Composting Process

The fate of ARGs is intricate in composting due to the composting conditions, external stress, and biological factors [1]. Based on redundancy analysis (RDA), the correlation between ARGs and environmental parameters, substances that can cause selection pressure (antibiotics and heavy metals), MGEs, and microbial communities were further dissected (Figure 6). Nine ARG subtypes (*floR*, *strB*, *tetW*, *tetM*, *tetA*, *dfrA1*, *cmlA5*, *sul1*, and *aac(6′)-Ib*) and nine dominated bacterial phylum, including Firmicutes, Proteobacteria, Actinobacteriota, Bacteroidota, Myxococcota, Cyanobacteria, Spirochaetota, Chloroflexi, and Desulfobacterota, were selected for analysis. As indicated by the length and angle of the variables in RDA, the most significant environmental factor determinant for the fate of ARGs was temperature. Temperature exhibited a correlation with all ARGs, indicating that high temperature was conducive to the removal of ARGs. Similar results were also obtained previously, leading to the conclusion that temperature played a key role in the fate of ARGs [36,44]. The primary reasons for this include the promotion of antibiotics, pesticides, and organic substance degradation at high temperatures, thereby reducing the selective pressure [22]. Additionally, high temperatures directly eliminate mesophilic and anaerobic bacterial hosts of ARGs and MGEs [45]. Moreover, thermophilic conditions weaken the horizontal gene transfer of ARGs by inhibiting MGEs [46]. The C:N ratio and TOC were positively correlated with most ARGs and negatively correlated with *sul1*, while pH had the opposite results. It was corroborated that $bla_{TEM\text{-}1}$ and bla_{AmpC} positively correlated with TOC during the composting of swine manure [47] and that some ARGs were negatively correlated with pH [44], particularly *tetW*, *tetM*, and *tetA* [36], while *sul1*, *sul2*, and $bla_{CTX\text{-}M}$ had a positive correlation with the pH value [9]. These results are consistent with this study.

The direct selection pressure induced by antibiotics and the synergistic selection pressure caused by heavy metals also contribute to the increase in ARG abundance in the environment that may further hinder the removal of ARGs [9,43]. In this study, external stress was mostly due to the presence of antibiotics. Antibiotics were significantly positively correlated with most ARGs, except *sul1* in CC. Moreover, the heavy metals Cu, Zn, As, and Pb had a positive relationship with *sul1* in the CC process. Lu et al. [48] found that the contents of Cu, Zn, and Pb were positively correlated with the abundance of the *sul1*, *sul2*, and *sul3* genes in an estuary. ARGs are carried by host bacteria and propagated through

MGEs; therefore, biotic parameters are strongly correlated with the variations in the ARG profiles during composting. MGEs showed a close positive correlation with most ARGs. The possible explanation for this is that MGEs and ARGs share the same host bacteria and MGEs are mainly responsible for ARG propagation [1]. Thus, inhibition of MGEs is essential for controlling the rebounding of ARGs. Positive correlations were observed between Cyanobacteria, Desulfobacterota, and Spirochaetota and most ARGs, which may reveal the potential host bacteria corresponding to ARGs.

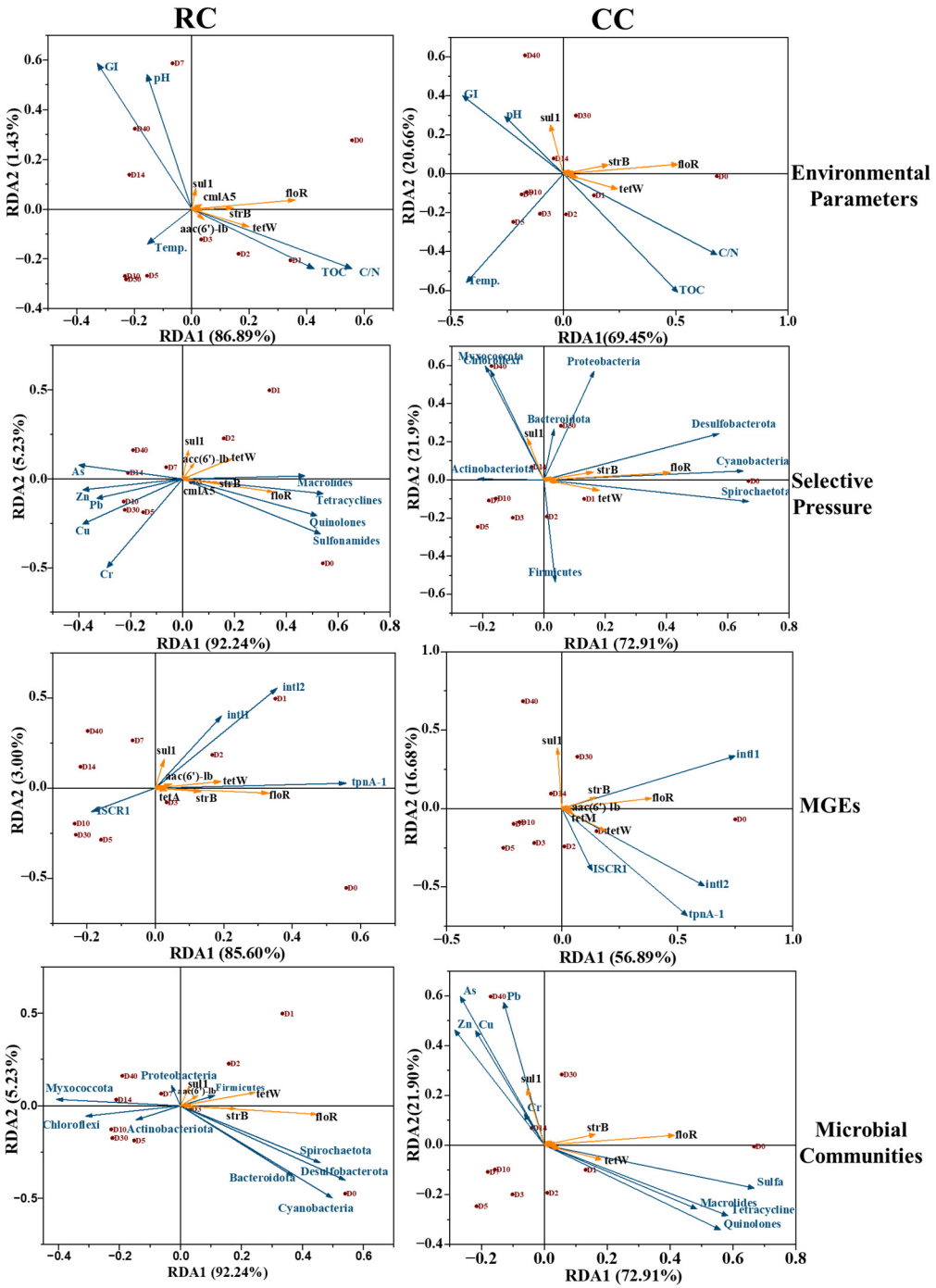

Figure 6. Effects of environment parameters, external stress, MGEs, and the microbial community on ARG variation patterns. Redundancy analysis (RDA) of the correlation between ARGs and environmental parameters, external stress, MGEs, and microbial communities.

Taking all biotic and abiotic factors into consideration, a structural equation model was established to further reveal their direct and indirect effects on the variation of ARGs. The mechanisms governing the fate of ARGs in both composting processes were complicated (Figure 7). Based on SEM analysis, we identified three factors that hindered the removal of ARGs. Firstly, while significant shifts in the environmental conditions during the initial week of composting can eradicate a substantial portion of the microorganisms, the presence of heat-resistant microorganisms shields ARGs, allowing their proliferation through vertical gene transfer [41,42]. Secondly, the process of MGE-mediated horizontal gene transfer remains active, facilitating the movement of ARGs, such as the *sul1* gene located within the 3′-conserved segment of *intI1* [14,44]. Thirdly, composting fails to eliminate heavy metals and fully degrade antibiotics, leading to gene selective pressure that targets and retains resistant genes [49,50]. All potential factors determined the variation pattern of ARGs, but the difference lies in the relative importance of these factors. SEM showed that MGEs had the strongest effect on ARG abundances in RC, while environmental parameters were the major determinant of the fate of ARGs in CC. However, the dominant direct effect on ARG variation in both processes was MGE abundance. This suggests that the low frequency of horizontal gene transfer directly limited the propagation of ARGs.

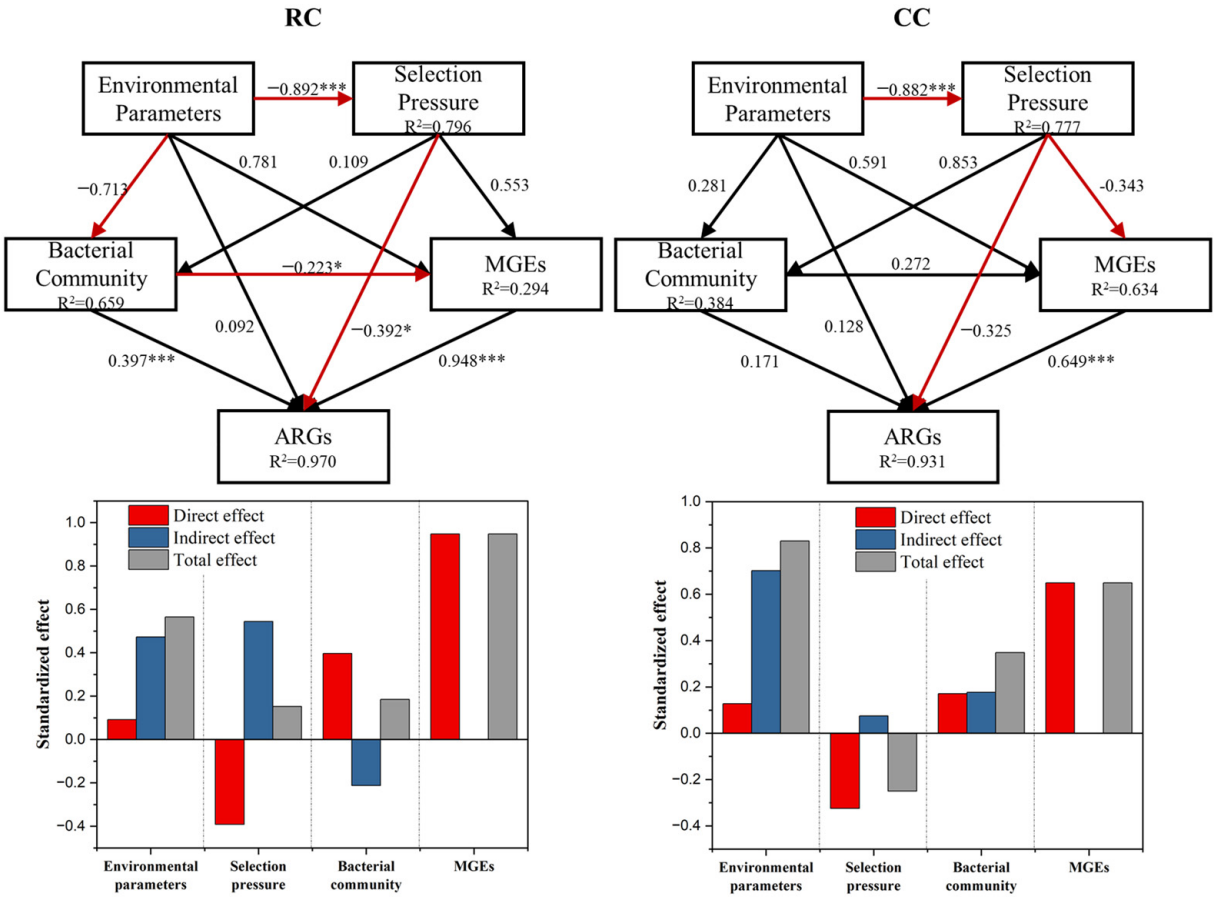

Figure 7. Structural equation models (SEMs) differentiated the effects of environmental parameters, selection pressure, the bacterial community, and MGEs on ARG patterns in both composting processes. Red and black arrows show negative and positive effects, respectively. * ($p < 0.01$), and *** ($p < 0.001$).

Temperature control significantly intensified the effect of environmental parameters on other potential factors, ultimately strengthening the control of the fate of antibiotics. Concretely, in addition to direct effects, temperature also enhanced the removal of MGEs and antibiotics and affected the microbial community structure. This indicates that tem-

perature control can inhibit ARG rebound in all aspects, which is a promising strategy for inhibiting the rebounding of ARGs in the late phase of composting.

4. Conclusions

In conclusion, this study demonstrated that RC treatment showed the maximum GI values of 93.28% on day 9, while CC had the highest GI of 80.54% on day 14. RC removed the greatest amount of the targeted antibiotics, 79.3% on day 40, while in CC this figure was only 75.26%. Among the targeted ARGs, the genes resisting β-lactamase and chloramphenicol were removed at 100% in RC and the overall reduction was 85% on day 40, while MGEs were reduced by 97%. In contrast, CC exhibited a reduction of 60% of ARGs and 85% of MGEs, while ARGs rebounded and were enriched by 8.2 times in CC by the end of the composting process. RDA and SEM analyses indicated that composting temperature was the key driver in suppressing the rebounding of ARGs in the late phase of composting in RC, whereas MGEs and enrichment of potential ARG hosts played a crucial role in the rebounding of ARGs in CC. Through adjusting the composting temperature, maximum ARG and antibiotic removal can be achieved while restricting their rebounding; however, additional research on the removal of ARGs is recommended to minimize and curb the risks associated with ARGs.

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