Effects of different composting methods on antibiotic-resistant bacteria, antibiotic resistance genes, and microbial diversity in dairy cattle manures

Minjia Tang,1 Zhongyong Wu,1 Wenzhu Li,2 Muhammad Shoaib,1 Amjad Islam Aqib,3 Ruofeng Shang,1 Zhen Yang,1 and Wanxia Pu1*

1Key Laboratory of New Animal Drug Project, Gansu Province/Key Laboratory of Veterinary Pharmaceutical Development, Ministry of Agriculture and Rural Affairs/Lanzhou Institute of Husbandry and Pharmaceutical Sciences of the Chinese Academy of Agricultural Sciences, Lanzhou 730050, China
2Qilhe District People’s Hospital of Lanzhou, Lanzhou 730050, China
3Department of Medicine, Cholistan University of Veterinary and Animal Sciences, Bahawalpur 63100, Pakistan

ABSTRACT

Composting is a common practice used for treating animal manures before they are used as organic fertilizers for crop production. Whether composting can effectively reduce microbial pathogens and antibiotic resistance genes remain poorly understood. In this study, we compared 3 different dairy manure composting methods—anaerobic fermentation (AF), static compost (SC), and organic fertilizer production (OFP)—for their effects on antibiotic-resistant bacteria, antibiotic resistance genes, and microbial community diversity in the treated manures. The 3 composting methods produced variable and distinct effects on antibiotic-resistant bacteria, antibiotic resistance genes, and microbial community diversity in the treated manures. The 3 composting methods produced variable and distinct effects on antibiotic-resistant bacteria, zoonotic bacteria, and resistance genes, some of which were decreased and others of which showed no significant changes during composting. Particularly, SC and OFP reduced chloramphenicol resistance gene fexA and opportunistic pathogen *Vibrio fluvialis*, whereas AF significantly reduced tetracycline resistance gene tetB and opportunistic pathogens *Enterococcus faecium* and *Escherichia fergusonii*. The compositions of microbial communities varied significantly during the composting processes, and there were significant differences between the 3 composting methods. In all 3 composts, the dominant phyla were *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria*. Interestingly, *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* remained stable in the entire AF process, whereas they were dominated at the beginning, decreased at the early stage of composting, and rebounded at the later stage during SC and OFP. In general, SC and OFP produced a more profound effect than AF on microbial community diversities, pathogens, and dominant species. Additionally, *Enterococcus aquimarinus* was isolated from AF for the first time. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States function prediction analysis indicated that the genes related to membrane transport and amino acid metabolism were abundant in the 3 composts. The metabolism of amino acids, lipids, and carbohydrates increased as composting progressed. The biosynthesis of antibiotics was enhanced after fermentation in the 3 composting methods, and the increase in the SC was the most obvious. These results reveal dynamic changes in antibiotic-resistant bacteria, antibiotic resistance genes, microbial community composition, and function succession in different dairy manure composts and provide useful information for further optimization of composting practices.

Key words: dairy manure compost, antibiotic-resistant bacteria, antibiotic resistance genes, microbial community diversity

INTRODUCTION

China has become one of the major livestock- and poultry-producing countries in the world. Because of the large production scale, more than 4 billion tons of livestock and poultry manures are produced and discharged every year (Han et al., 2021). It is estimated that by 2050, the livestock and poultry manures in China will exceed 8 billion tons per year, and a considerable part thereof will be used as fertilizer to improve the organic matter and nutrient contents of soils in agriculture (Zhang et al., 2019b). The use of organic fertilizer in farmland is expected to change the structure of the soil microbial communities and improve enzymatic activities (Lazcano et al., 2012). However, using untreated manure as fertilizer will increase the microbial burden on the farm due to the complex composition of manures, and many of these microbials will flow into the river via runoff (Grewal et al., 2006; McLaughlin et al., 2009). Also, pathogens and antimicrobial residues in untreated manures may promote the
transfer of harmful bacteria to crops that will enter the food chain for human consumption (Klerks et al., 2007; Erickson et al., 2014). For example, published studies have shown that organic fertilizers can introduce antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARG) to the soil (Cunningham et al., 2020), and endophytic bacteria that harbor ARG can colonize plants and may persist throughout the vegetable growth stage (Pu et al., 2019). According to the World Health Organization’s statistics, there are about 1.5 billion diarrheal cases worldwide each year, 70% of which are caused by food contaminated with pathogenic microorganisms (Akeda, 2015). Delahoy reported that most pathogens in food might be originated from livestock excretions (Delahoy et al., 2018). At least 150 species of human or animal pathogens have been identified in animal manures (McDaniel et al., 2014), which may survive in water, soil, and other environments for a long time and transmit to humans through crops or animal products.

Antimicrobial agents are widely used in the livestock and poultry industry for prophylaxis and treatment of infectious diseases and even for promoting growth (Sun et al., 2017). The overuse and inappropriate use of antimicrobial agents have driven the rapid evolution of ARB and ARG, reducing the therapeutic potential of antibiotics against human and animal pathogens (Wright, 2010). Antibiotics in the environment may provide a selective advantage to ARB, resulting in their proliferation and expansion in the environment (Bengtsson-Palme et al., 2018). It is worth noting that many antimicrobials used for animal agriculture are also important for human medicine. For example, macrolides are considered “highest priority and critically important antimicrobials” by the World Health Organization, and previous-generation cephalosporins, lincosamides, and sulfonamides are classified as “highly important,” all of which are regularly used in food animal production. A study showed that the ARG in swine farms were closely associated with the residues of sulfonamides, quinolones, and tetracyclines (Zhu et al., 2013). Numerous studies demonstrated that crops fertilized with dairy manures could accumulate antibiotic residues (Tasho and Cho, 2016; Pan and Chu, 2017a, b) and ARG (Marti et al., 2013; Marti et al., 2014; Tien et al., 2017). Due to the extensive use of antibiotics in livestock and poultry production systems, ARG were increasingly detected in livestock and poultry manures and may disseminate to other environmentally indigenous bacteria through horizontal gene transfer (Yang et al., 2014), creating a reservoir of ARG and ARB that poses a threat to both public and animal health.

To reduce pollution, the Chinese government has recently stipulated that the manures of farm animals be treated innocuously before they are reused and discharged (Zhang et al., 2021b). However, it is unknown whether manure treatments can effectively control the discharge of microbials and whether they can efficiently reduce ARB and ARG. To address these questions, we examined the ARB, ARG, and microbial diversity of 3 different dairy manure compost treatments in northwest China.

MATERIALS AND METHODS

Ethics

This study did not involve endangered or protected species, animals, or clinical experiments.

Sample Collection

Three composting methods, anaerobic fermentation (AF), static compost (SC), and organic fertilizer production (OFP), were examined in this study because they are commonly used for treating cattle manure in China (Meng et al., 2019; Li et al., 2020; Zhang et al., 2021a). The sampled dairy farms were located in Xinjiang Province and Qinghai Province of northwest China. All samples were collected in mid-March, and at that time the average temperature in both locations was around 10°C and the average daylight was 11 to 12 h. Compost samples were collected at 5 different sites corresponding to different stages of each composting method (discussed next). At each site, triplicate samples were obtained and each sample was collected by the 5-point sampling method (Zhou et al., 2021). In total, 45 samples were collected from the 3 composting methods.

At the Xinjiang site, composting was done by AF. Briefly, the raw manure (Z1) was separated into precipitated sand and suspension by stirring, suspending, and precipitating. The precipitated sand (Z2) was used as bedding for the dairy farm after more than 20 d of sun exposure, and the suspension was put into the fermenter for fermentation. After fermentation, products were separated into 3 parts. The air portion was used as bioenergy, the solid part (Z3) was used as bedding after sun exposure for more than 1 mo (Z4), and the liquid part (Z5) was used as fertilizer (Supplemental Figure S1A, https://doi.org/10.7910/DVN/WHS3EA; Tang et al., 2022). Samples were collected at the stages of Z1, Z2, Z3, Z4, and Z5 for various analyses.

The composting methods used at the Qinghai site were SC and OFP. For SC, raw manure from cowshed bedding (S9) was mechanically pressed and separated into liquid sewage (SS) and solid residue (S10). The liquid sewage was discharged into the lagoon and ir-
rigated to the farm fields after over 1 mo of storage and natural fermentation. The solid residue was piled up and used as bedding for the dairy farm after more than 20 d of SC and sun exposure (S12) (Supplemental Figure S1B). During the SC, the pile was turned once every 7 d and the temperature in the manure pile was between 45°C and 50°C. The collected samples included raw manure (S9), liquid sewage in the lagoon (SS), solid residue (S10), and solid after 10 d (S11) and 20 d (S12) composting and sun exposure.

For OFP, the raw manure, which was mainly from the playground and bedding (S13), was mixed with rice husk powder. The mixture (S14) was then put into a manure pit. For every 5 tons of mixed manure materials, 1 kg of fermentation agents was added, which contained approximately 1 billion viable bacteria per gram. The viable bacteria mainly included Myceliophthora thermophila, Streptomyces thermophilus, Bacillus stea- rothermophilus, Bacillus pallidus, and Bacillus subtilis. During the 1-mo fermentation period, the composts were turned and the temperature was measured every day. The fermentation temperature within the pit was approximately 60°C. After fermentation (S15), the compost material was crushed and screened, then it was combined with chemicals including nitrogen, phosphorus, and potassium to the total content of 4% (S16). The final product was processed into granular fertilizer after drying, deducting, and cooling and was used as organic fertilizer (S17) (Supplemental Figure S1C). Samples of OFP were collected at S13, S14, S15, S16, and S17. For all 3 composting methods, a clear sampling scheme and sample information are shown in Supplemental Figure S1 and Supplemental Table S1 (https://doi.org/10.7910/DVN/WH33EA; Tang et al., 2022).

**Detection of ARG in Manures by PCR**

The 3 samples from each sampling site were mixed, and the DNA of solid and liquid samples was extracted with the soil extraction kit (OMEGA Soil DNA Kit: D5625-01) and the liquid extraction kit (OMEGA Water DNA Kit: D5525-01) according to the manufacturer’s instructions, respectively. In total, 32 ARG were analyzed by PCR including chloramphenicol/florfeni- col resistance genes (floR and fexA), fluoroquinolones resistance genes [gyrA, gyrB, grlA, grlB, aac(6’)-Ib-cr, and qnrA], β-lactam resistance genes (mecA, mecc, and femA), glycopeptide resistance genes (vanA, vanB, and vanC), sulfonamides resistance genes (sulf1, sulf2, and sulf3), aminoglycoside resistance genes aac(6’)-aph(2’), macrolides resistance genes (ermA, ermB, ermC, and ermF), tetracycline resistance genes (tetA, tetB, tetC, tetD, and tetM), carbapenem resistance genes (blaKPC and blaSDM1), and multidrug resistance genes (norA, cfrB, and cfrC) (Liu et al., 2009a, 2018; Wang et al., 2014a,b; He, 2016; Yang et al., 2016; Zhang et al., 2019a, 2020a; Chen et al., 2020; Meng et al., 2020; Shen et al., 2020; Zhou et al., 2020). Primer sequences and PCR reaction conditions are displayed in Supplemental Table S2 (https://doi.org/10.7910/DVN/WH33EA; Tang et al., 2022).

**Isolation and Identification of Cultivable ARB**

To a 500-mL conical flask containing 450 mL of 0.9% saline, a 50-g solid compost sample was added and mixed thoroughly. From the suspensions, 500 µL was plated onto an antibiotic-containing Luria-Bertani (LB) plate and triplicate plating was used for each sample suspension. The plates were aerobically incubated at 37°C for 18 to 24 h. The antibiotics used in the LB plates included kanamycin, oxacillin, sulfamethoxazole-trimethoprim (SXT), erythromycin (ERY), tetracycline (TET), vancomycin (VAN), florfenicol, and ciprofloxacin, and their concentrations were 64, 4, 8, 16, 32, 8, and 4 µg/mL, respectively, based on the breakpoints defined by the Clinical and Laboratory Standards Institute (2019). According to visual observation, colonies with different colors, morphology, and opacity were isolated from the antibiotic-containing LB plates and subcul- tured onto LB plates until the colony morphology on the same plate was consistent. The isolated strains were identified by 16S rDNA sequencing using the universal bacterial primers 27F (5’-GAGAGTTTGATCCTG-GCTCAG-3’) and 1492R (5’-CTACGGCTACCTTGT-TACCAG-3’) (Popović et al., 2013). Briefly, the total bacteria DNA of each isolate was extracted with the TIANamp Bacteria DNA Kit (Tiangen) following the manufacturer’s instructions. Bacterial 16S rDNA was amplified by PCR and sequenced in Shanghai Person- albio Technology. The results of the sequence alignment were analyzed by the Basic Local Alignment Search Tool of the National Center for Biotechnology Information database.

**High-Throughput Sequencing Analysis**

The V3–V4 regions of bacterial 16S rDNA genes were amplified by primers 338F (5’-ACTCTACGGGAGGCAGCA-3’) and 806R (5’TACACTACHVGGGTWTCTAAT-3’) with the barcode (Yang et al., 2020). The TruSeq DNA PCR-Free Sample Preparation Kit (Illumina), Qubit® 2.0 Fluorometer (Thermo Scientific), Agilent Bioanalyzer 2100 system, and Illumina NovaSeq platform were used to build sequencing libraries, evaluate the library quality, and sequence the library. Sequence analysis was performed using Uparse soft-
ware (Uparse v7.0.1001, http://drive5.com/uparse/), and MUSCLE software (version 3.8.31, http://www.drive5.com/muscle/) was used for multiple sequence alignment to determine the phylogenetic relationship of different operational taxonomic units and distinguish the dominant species. The α-diversity indices were calculated by the QIIME program (version 1.7.0) and visualized by R software (version 2.15.3). The β-diversity of weighted and unweighted UniFrac was calculated by QIIME software. Unweighted pair-group method with arithmetic means clustering was performed as a hierarchical clustering method to interpret the distance matrix using average linkage and was conducted by QIIME software. The linear discriminant analysis effect size (LEfSe, http://huttenhower.sph.harvard.edu/galaxy/) approach was used to determine the taxa that were enriched in a particular environment. The top 50 genera with the highest abundance were clustered, and their heatmaps were drawn by the vegan R package. Nonmetric multidimensional scaling was applied to analyze community variation with UniFrac distances. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt, http://huttenhower.sph.harvard.edu/galaxy/tool_runner?tool_id=PICRUSt_normalize) was used for predicting the metabolic function of the bacterial community.

RESULTS AND DISCUSSION

Distribution of Cultivable ARB and Related ARG in the 3 Composts

The PCR data showed that SC reduced the chloramphenicol resistance gene \textit{fexA} and fluoroquinolone resistance gene \textit{gyrB} (Figure 1A). The OFP method reduced the chloramphenicol resistance gene \textit{fexA}, TET resistance gene \textit{tetB}, and multidrug resistance gene \textit{cfrB}. The \textit{gyrB}, \textit{aac(6')-Ib-cr}, \textit{cfrC}, and \textit{ermA} genes existed in the OFP samples at the beginning, disappeared during processing, but rebounded at the later stage. These changes could be explained by differential death and survival of ARB and horizontal gene transfer of ARG between bacteria (Miller et al., 2016; Sun et al., 2016) (Figure 1A). Anaerobic fermentation produced an obvious reduction in TET resistance gene \textit{tetB} and fluoroquinolone resistance genes (\textit{gyrB} and \textit{grlB}). Interestingly, \textit{ermA}, \textit{tetC}, and \textit{cfrB} first disappeared and then reappeared (Figure 1A). Previous studies showed that AF produced certain effects on ARB and ARG in livestock manures, but the specific effects were not consistent. Beneragama et al. (2013) found that mesophilic AF reduced the proportion of cefazolin-resistant bacteria and corresponding ARG. Sun et al. (2016) found that thermophilic AF could effectively reduce the relative abundance of ARG in cow dung, but Huang et al. (2019) showed that high temperature did not always eliminate ARG. Sharma et al. (2009) showed that most TET and ERY ARG were stable over a 126-d compost, whereas some ARG increased. In contrast, Walczak and Xu (2011) found a significant reduction of ARG during cattle manure composting. Other recent studies have noted significant reductions in the diversity and relative abundance of ARG and mobile genetic elements in actively managed and aerated cattle manure composts (Gou et al., 2018).

The plating results showed that all samples yielded colonies on the VAN-containing plates, and species isolated from them were gram-negative bacteria (Figure 1B). It is probably because VAN is not effective against gram-negative bacteria (Wishart et al., 2008). The isolation rates of ARG on kanamycin, ERY, SXT, and oxacillin plates were also relatively high. This is consistent with the result of ARG detection, as the detection rates of \textit{aac(6')}/\textit{aph(2′)I}} that mediate kanamycin resistance and \textit{ermABC} that mediate ERY resistance were high (Figure 1A). However, genes \textit{mecA}, \textit{mecC}, \textit{femA}, \textit{bla}_{\text{KPC}}}, and \textit{bla}_{\text{NDM-1}} mediating β-lactam resistance and genes mediating SXT resistance were not detected by PCR. This could be explained by the possibility that the oxacillin and SXT resistance phenotypes were due to resistance mechanisms that were not targeted by the ARG-detecting PCR primers used in this study. The isolation rates of ARG on ciprofloxacin-, florfenicol-, and TET-containing plates were relatively low, suggesting that resistance to these antibiotics was low in the analyzed samples (Figure 1A, B).

Single colonies identified from the 3 types of composts were classified into 64 different bacterial species, belonging to 27 genera. The majority of the isolated strains belonged to the genera \textit{Bacillus}, \textit{Enterococcus}, and \textit{Pseudomonas} (Figure 1C, D, and E). As shown in Figure 1C, \textit{Alcaligenes faecalis} was the most abundant, followed by \textit{Enterococcus aquimarinus} and \textit{Alcaligenes aquatilis}, during the AF process. \textit{Escherichia fergusonii} was the most abundant, followed by \textit{A. faecalis}, during the SC process (Figure 1D). \textit{A. faecalis} was the most abundant, followed by \textit{E. fergusonii} and \textit{A. aquatilis}, during the OFP process (Figure 1E).

\textit{Alcaligenes faecalis} existed in almost all samples analyzed in this study, which is in line with the findings reported by Zhong et al. (2020b). Interestingly, the OFP composting significantly decreased its isolation rate (Figure 1C–E). This bacterium widely exists in natural environments (Majewski et al., 2020) and is considered an opportunistic pathogen highly resistant to antibiotics and a plant probiotic bacterial endophyte (Tena et al., 2015; Hasan et al., 2019; Puah et al., 2019; Ngbede et al., 2020). Recent studies have shown that
A. faecalis can degrade a variety of toxic and harmful substances such as phenol (Jing et al., 2007), cyanide (Boulanger and Murphy, 2003), and hydrogen sulfide. Numerous studies reported E. fergusonii as a newly identified human and animal pathogen that shows extensive resistance to antibiotics (Lagacé-Wiens et al., 2010; Forgetta et al., 2012; Glover et al., 2017; Adesina et al., 2019) and is commonly isolated from livestock manures (Herráez et al., 2005; Hariharan et al., 2007; Oh et al., 2012), beef, and cheese (Fegan et al., 2006). This species existed in the raw manure samples (Z1, S9, S13) and disappeared in the later stages of AF and OFP (Figure 1C–E), suggesting that the composting treatments can effectively reduce opportunistic pathogen E. fergusonii. This finding is notable, as little is known about the effect of composting on E. fergusonii. Enterococcus aquimarinus existed in almost all samples of AF (Figure 1C–E) and E. faecium, Enterococcus faecalis.
Staphylococcus lentus, Vagococcus fluvialis, Vagococcus lutrae, Klebsiella pneumoniae, Pseudomonas aeruginosa, Acinetobacter lwoffii, Raoultella terrigena, and Microbacterium paraoxydans. Among these opportunistic pathogens, Enterococcus faecium decreased in the late stage of AF, and V. fluvialis decreased in the late stage of SC and OFP. There was no significant difference in other opportunistic pathogens among the 3 composting treatments (Figure 1C–E).

Most of the isolated species were nonpathogenic, and some even contributed to plant growth by providing protection against the hypersaline environment or enhancing the usability of nutrients. Thermophilic bacterium A. aquatilis is rare but was with a high isolation rate in the 3 composting methods in this study (Figure 1C–E). Alcaligenes aquatilis has a wide range of beneficial effects (Haouas et al., 2021). It can use aromatic hydrocarbons as sources of carbon and nitrogen (Durán et al., 2019) and dissolve inorganic phosphates, which contributes to the growth of maize plants (Pande et al., 2017). Other beneficial bacteria were also detected in this study, such as Staphylococcus equorum, Achromobacter piechaudii, Enterobacter ludwigii, and Enterobacter tabaci, which can enhance the tolerance of plants to salt (Mayak et al., 2004; Lee et al., 2018; Khan et al., 2020). These species were only detected in SC and OFP (Figure 1D, E), suggesting that these 2 treatments were conducive to the growth or survival of high-salt-tolerant species. Some bacteria that can remove heavy metals or provide plants with the ability to resist heavy metals were analyzed in the present study, including Pseudomonas tuomuerensis (Zhang et al., 2012), Providencia vermlicola (Tan et al., 2020), Citrobacter murliniae (Boechat et al., 2017), Pseudomonas indolozydans (Shahid et al., 2020), Enterobacter ludwigii (Wang et al., 2020), Bacillus wiedmannii (Chen et al., 2018), and Proteus hauseri (Khallilian et al., 2015). These species were most commonly distributed in SC, followed by OFP and AF (Figure 1C–E). Some bacteria detected in this study possess biodegradation functions. For example, Pusillimonas norremannii can degrade substituted salicylates (Stolz et al., 2005), Enterococcus mundtii can convert hexose and pentose sugars (Collins et al., 1985), and Enterobacter ludwigii can biodegrade and detoxify chlorimuron-ethyl (Pan et al., 2018) (Figure 1C–E).

Based on the results from this study, there were considerable variations and changes in ARG associated with the 3 composting methods. According to the bacterial isolation result, AF and OFP significantly reduced opportunistic pathogen E. fergusonii. Additionally, AF significantly reduced opportunistic pathogen Enterococcus faecium, and SC and OFP significantly reduced opportunistic pathogen V. fluvialis. However, changes in ARB and ARG across the 3 composting methods were not uniform, and it is difficult to state which method is superior to the others. Variables other than compost management also affect compost resistomes and their fate. These include the concentration of residual antibiotics, the co-occurrence of ARG and mobile genetic elements, and growth-modulating factors such as nutrient and heavy metal contents, which may influence microbial population shifts and gene regulation and transfer (Qian et al., 2016, 2018). Additionally, the inability to correlate ARB and ARG could be related to the timing of sampling (Singer et al., 2008; Chambers et al., 2015), strain level differences (McConnel et al., 2016), and horizontal gene transfer complexities (Dolejska et al., 2011; Gonggrijp et al., 2016). However, because the detection of ARG in this experiment was carried out by a limited number of PCR detections, it was not sufficient to detect all changes in ARG during the composting processes. Another limitation of the present study was that ARB were only obtained by aerobic culture, not other culture conditions. Therefore, it is likely that the number of different ARB identified in the composts was underestimated and incomplete.

**Sequencing Analysis**

High-throughput sequencing of bacterial 16S rDNA (V3-V4 region) was performed to determine the differences and changes in microbial community diversity (MCD) of the 3 different manure composts. A total of 1,069,318 high-quality sequences were obtained and were classified as 5,790 operational taxonomic units, which were classified into 985 phyla, 983 classes, 958 orders, 800 families, 383 genera, and 40 species on average. The average high-quality sequences generated per sample were 71,288 for the bacterial community. The sequence information of each sample was shown in Supplemental Table S3 (https://doi.org/10.7910/DVN/WHS3EA; Tang et al., 2022).

**MCD**

The rarefaction curve based on the Chao1 index and observed species shows that all the curves tend to be flat in the current sequencing amount, and the sequencing depth is sufficient to capture the MCD in the samples (Figure 2A, B). The α diversity index for each manure compost stage was compared. Chao1 and Shannon diversity indices are important to measure distribution richness and evenness of species in compost ecology (Huang et al., 2013; Du et al., 2019).

As Figure 2C–D shows, the Chao1 and Shannon indices decreased first and then increased continuously during the 3 compost treatments. The lowest points of
AF, SC, and OFP were Z2, S11, and S15, respectively. This indicated that the microbial community richness and evenness of the solid after solid-liquid separation (SLS), the solid after 10 d of compost, and the solid after fermentation were the lowest during the 3 composting processes. The α-diversity results showed that there was no significant difference in richness and evenness of microbial communities at different stages of AF (P > 0.05), indicating that microbial dynamics and abundance are no different throughout the AF process, which is consistent with the findings by Resende et al. (2016). On the contrary, the richness and evenness of microbial communities after fermentation during SC and OFP were significantly reduced (P < 0.05). Both processes were in the early stages of composting, when the temperature was relatively high. The variation trend was consistent with the notion that the quantity and diversity of community would decrease significantly with the increase of temperature and would increase with the depletion of organic matter (Tian et al., 2013). We also noticed that the richness and diversity of the microflora increased after a long period of storage (Figure 2C, D). This might be due to the presence of degraded organic substances that promoted the rapid evolution of microbes and the decrease of temperature in the later compost stage (Gannes et al., 2013; Meng et al., 2019; Sun et al., 2019).

Figure 2. Rarefaction curves and α diversity index in the 3 composts. (A) Chao1 richness. (B) Observed species. (C) Chao1 index. (D) Shannon index. Z1, Z2, Z3, Z4, and ZS are the sampled sites for anaerobic fermentation; S9, S10, S11, S12, and SS are the sampled sites for static compost; and S13, S14, S15, S16, and S17 are the sampled sites for organic fertilizer production. Three samples were collected from each site, and each data point is shown as the mean ± SD (n = 3).
Nonmetric multidimensional scaling was conducted based on weighted UniFrac to elucidate the differences in microbial composition among different manure composts. Samples after AF (Z2, Z3, and Z4) were still close to the raw manure sample (Z1), indicating that AF had little effect on the MCD (Figure 3), which is similar to observations made by Wei and Guo (2018). On the other hand, our data showed that the distance matrix of the samples after SC (S11 and S12) was far from the samples before fermentation (S9 and S10), and the distance matrix of the samples in OFP showed the same trend (Figure 3). These results demonstrated that the SC and OFP composting had a greater influence on MCD. Given the ecological and physiological diversity in microbial communities (Delgado-Baquerizo et al., 2017; Mooshammer et al., 2017), various species have different ecological and physiological characteristics and respond to compost in different ways. Therefore, distinct microbial taxa predominated different compost piles, and each member adapted to specific environmental conditions that varied at different stages (Zhao et al., 2018; Qiao et al., 2019; Wu et al., 2020). In addition, the feed may affect microbial diversity in manure. Background information showed that although the total nutritional values of feed formula in the 2 locations were roughly the same, the proportions of various feed ingredients were different. The proportions of cottonseed meal and silage alfalfa were higher in the feed used for Xinjiang dairy farms, whereas the proportions of soybean meal and whole-crop corn silage were higher in the feed used for Qinghai dairy farms, which may also account for some of the differences in microbial compositions after fermentation.

To determine the functional communities in samples, LEfSe was applied to identify the distinct groups across the different compost treatments, which are shown in a cladogram (Figure 4). The bacterial taxa varied during the 3 compost processes. There were 15 significantly abundant bacterial taxa, including *Armatimonadetes*, *Actinobacteria*, *Acidobacteria*, TM7, and *Alphaproteobacteria*, in the SC process. Phylum TM7 had only 16S rDNA sequence characteristics without known pure culture representatives but is widely distributed in the environment (Hugenholtz et al., 1998, 2003). Studies have shown that TM7 is associated with human inflammatory mucosal diseases (Marcy et al., 2007; Kuehbacher et al., 2008) and oral disease (Brinig et al., 2003). The presence of TM7 in the SC suggests that it poses a potential health risk when used as farm fertilizer. There were 3 bacterial taxa including CFB_26 (order) that were significantly abundant in the OFP process. Meanwhile, only one taxon, *Deltaproteobacteria*, was enriched in AF. We also carried out LEfSe analysis (linear discriminant analysis score greater than 2) among each stage of AF, SC, and OFP. No biomarkers were identified between stages of the different compost processes (Figure 4).

**Taxonomic Analysis of Microbial Composition Profiles**

### Microbial Community Composition at the Phylum Level

Seventeen known phyla were identified in all samples: *Firmicutes*, *Proteobacteria*, *Chloroflexi*, *Actinobacteria*, *Bacteroidetes*, *Gemmatacomadetes*, *Cyanobacteria*, *Synergistetes*, *Acidobacteria*, [Thermi], *Verrucomicrobia*, *Spirochaetes*, *Tenericutes*, *Planctomycetes*, *Nitrospirae*, *Chlorobi*, and *Fibrobacteres* (Supplemental Figure S2, [https://doi.org/10.7910/DVN/WH53E3A](https://doi.org/10.7910/DVN/WH53E3A); Tang et al., 2022). The major phyla among the microbial communities in the 3 composting methods were *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Chloroflexi*, and *Bacteroidetes*. Numerous studies have shown that they are related to the degradation of lignocellulose (Gannes et al., 2013; Zhang et al., 2015; Awasthi et al., 2017; Meng et al., 2019). *Firmicutes* were the dominant microflora in anaerobically fermented solids, which are most common in biogas reactors and are responsible for organic degradation and fermentation (McGarvey et al., 2007; Liu et al., 2009b;
Kampmann et al., 2012; Bengelsdorf et al., 2013). Actinobacteria play a dominant role in the late compost stage, which can release inorganic nutrients related to humus formation and may inhibit pathogens by secreting antibiotics, as well as play an important role in the degradation of cellulose, hemicellulose, lignin, and chitin (Steger et al., 2007; Franke-Whittle et al., 2009; Neher et al., 2013). Proteobacteria are environmental organisms that have no relationship with animal diseases (Coenye et al., 2005). Firmicutes and Actinobacteria were usually higher in compost piles, suggesting that compost fertilization might transmit Firmicutes and Actinobacteria into soils (Sivasakthi et al., 2014; Chowdhury et al., 2015; Rybakova et al., 2016; Shafi et al., 2017; Chaurasia et al., 2018).

There were differences in dominant phyla at different stages among the 3 composting methods. As shown in Supplemental Figure S2, Firmicutes and Proteobacteria were always dominant in the AF process. Both of them were dominant in samples before fermentation and showed a downward trend at the early stage but rebounded at the later stage during SC and OFP. Chloroflexi were dominant in samples after fermentation during SC and OFP. Actinobacteria showed a downward trend in the late stage of SC but remained stable in OFP. Bacteroidetes were stable during the AF process and decreased during the SC and OFP processes but rebounded at the later stage of SC. A possible explanation is that the manure temperature was high at the early stage of compost, which could inhibit the growth of Proteobacteria, Firmicutes, and Bacteroidetes (Chroni et al., 2009). However, Chloroflexi was ubiquitous in the composting process (Tian et al., 2013), and its relative proportion increased when the abundance of other bacteria decreased. Firmicutes and Proteobacteria increased at various levels at the later stage of SC and OFP, when the temperature of the compost decreased (Supplemental Figure S2). Another note is that the content of Gemmatimonadetes increased by approximately 20 times in the later stage of OFP. The sharp
increase of *Gemmatimonadetes* was possibly due to the decrease of moisture in the compost, which is consistent with the report by DeBruyn et al. (2011).

**Microbial Community Composition at the Genus Level.** The abundance of genera in each sample is shown in Figure 5. The relatively abundant genera of the samples before fermentation (Z1, S9, S13, and S14) included *Facklamia*, *Unclassified_Aerococccaeae*, *Unclassified_Lachnospiraceae*, *Erysipelothrix*, *Unclassified_Ruminococcaceae*, *Unclassified_Erysipelotrichaceae*, *Unclassified_Porphyrmonadaceae*, *Psychrobacter*, and *Unclassified_[Tissierellaceae].* In Z2 and S10, the relatively abundant genera included *Unclassified_Pseudomonadaceae*, *Unclassified_Morazellaceae*, *Corynebacterium*, *Acinetobacter*, and *Arthrobacter*. The abundance of *Unclassified_Peptostreptococcaceae, Clostridium*, *Unclassified_Clostridiaeae, Sedimentibacter*, and *Clostridiales* was relatively high in ZS and SS. Notably, the clustering relationship of S9 and S10 is close, and so is that of S11 and S12. The same clustering relationship was also found in OFP. Those results indicate that SC and OFP yielded a major impact on dominant species. We also observed that the abundant genera of OFP changed significantly after fermentation, which may be due to the addition of viable bacteria in the compost, indicating that the addition of live bacteria can produce more obvious effects on fermentation and abundant genera. Other studies also showed that the addition of thermophilic bacteria and *Bacillus* were beneficial to the decomposition of litter and improved the fermentation process (Shen et al., 2019; Huang et al., 2021).

*Facklamia* is a bacteremia-related pathogen (Gahl et al., 2020) and can also lead to joint infection (Corona et al., 2014). *Rhusiopathiae* in *Erysipelothrix* is potentially zoonotic and can be transmitted to humans, causing erysipelas (Öppenraat et al., 2020). *Porphyromonas* in *Porphyromonadaceae* is a periodontitis-related pathogen (Zhang et al., 2020b). *Psychrobacter immobile* is an opportunistic pathogen that has been found in eyes, brain tissue, urethra, cerebrospinal fluid, and blood (Shao et al., 2021). All these species existed in the raw manure, indicating there are many zoonotic or opportunistic pathogens in untreated manure. If untreated manure is used as an organic fertilizer on farmland, the persistence of these bacteria in soil may enhance the likelihood of these bacteria entering the food chain through contaminated crops and becoming active ARG donors (Leclercq et al., 2016). *Pseudomonas* in *Pseudomonadaceae* is a pathogen associated with septicemia and lung infections, *Acinetobacter* causes nosocomial infection, and *Corynebacterium* is a potential human pathogen (Soltan Mohammadi et al., 2013). These species declined at the late stage of compost, suggesting that the 3 composting practices examined in this study were able to reduce these pathogens, which is in line with the findings by Li et al. (2019). However, pathogens such as *Unclassified_Porphyrmonadaceae*, *Unclassified_Erysipelothrichaceae*, and *Unclassified_[Tissierellaceae]* remained in the liquid after AF. If the liquid is used to fertilize farmland, pathogens may flow into the environment, which might pose direct or indirect threats to human and animal health. In general, the 3 composting methods yielded a certain level of reduction in pathogens, and the effect of AF was weaker than that of SC and OFP (Figure 5).

**Prediction of Functional Profiles of the Bacterial Community.** PICRUSt is designed to estimate the gene families contributed to a metagenome by bacteria or archaea identified and to predict functional profiles of bacterial communities based on 16S rDNA sequences (Langille et al., 2013). We performed PICRUSt to analyze the differences in bacterial functions among 3 manure composts. A total of 328 Kyoto Encyclopedia of Genes and Genomes pathways were obtained in the 3 different composts by comparing with the Kyoto database. Among these pathways, gene sequences in the 3 treatments were primarily involved in metabolism, followed by genetic information processing and environmental information processing. In addition, the analysis of the second functional layer of the predicted genes showed that it consisted of 41 subfunctions, and the gene abundance of membrane transport, replication and repair, carbohydrate, energy, and amino acid metabolism was high among the 3 composting methods (Supplemental Figure S3, https://doi.org/10.7910/DVN/WHS3EA; Tang et al., 2022), which is consistent with results reported by others (Bello et al., 2020; Liang et al., 2020). It is worth noting that the abundance of amino acid and carbohydrate functional genes was predominant among the 3 compost treatments, and the metabolism of amino acids, lipids, and carbohydrates increased as the composting process advanced. It is reported that amino acid metabolism promotes the growth and activity of bacteria by providing carbon and energy sources (López-González et al., 2015; Liang et al., 2020). Membrane transport and carbohydrate metabolism increased, whereas amino acid and energy metabolism remained stable after fermentation during SC and OFP (Supplemental Figure S3). This was probably owing to the degradation of energy-rich wastes contained in the compost, including crude proteins and sugars (Zhong et al., 2020a). The metabolism of carbohydrates may produce various compounds through cellulose and hemicellulose degradation (Toledo et al., 2017). Additionally, amino acids serve as a carbon and energy source for bacterial metabolism, which is con-
Figure 5. Heat map of bacterial community compositions at the genus level ($n = 3$ per sampled site). (A) Anaerobic fermentation (AF). (B) Static compost (SC). (C) Organic fertilizer production (OFP). Green represents low abundance, and red represents high abundance. The lines on the left of each panel represent the clustering relationship at the genus level, and the lines on top of each panel represent the clustering relationship of samples collected at different composting stages with unweighted pair-group method with arithmetic means clustering. Only the most abundant taxa (top 50 genera) are displayed. Z1, Z2, Z3, Z4, and ZS are the sampled sites for AF; S9, S10, S11, S12, and SS are the sampled sites for SC; and S13, S14, S15, S16, and S17 are the sampled sites for OFP.
The 3 composting methods examined in this study produced variable and distinct effects on ARB, zoonotic bacteria, and ARG as well as microbial communities. Some ARB and ARG decreased, whereas others showed no significant difference during the composting processes. All 3 composting methods reduced the richness and evenness of microbial microflora. Among the 3 composting methods, the effects of SC and OFP on MCD, including zoonotic and opportunistic pathogens, and dominant species were more profound than those of AF. The addition of viable bacteria can produce more obvious effects on fermentation and abundant genera. As shown by PICRUSt function prediction analysis, the genes encoding membrane transport, carbohydrate metabolism, and amino acid metabolism were abundant among the 3 composting methods. The biosynthesis of antibiotics was enhanced after fermentation in the 3 composting methods, and the increase in SC was the most obvious. These results provide useful information for further optimizing composting practices.

ACKNOWLEDGMENTS

This work was supported by the National Key Research and Development Program (2016YFD0501305) and the Agricultural Science and Technology Innovation Program of the Chinese Academy of Agricultural Sciences (25-LZIHPS-03). Wanxia Pu: conceptualization, writing—review and editing, funding acquisition. Minjia Tang: data curation, writing—original draft and formal analysis. Zhongyong Wu: conceptualization, investigation, formal analysis. Wenzhu Li: investigation. Muhammad Shoaib: investigation. Amjad Islam Aqib: writing—review and editing. Ruofeng Shang: writing—review and editing. Zhen Yang: writing—review and editing. The authors have not stated any conflicts of interest.

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ORCIDS

Minjia Tang https://orcid.org/0000-0002-7156-954X
Zhongyong Wu https://orcid.org/0000-0001-8946-8993
Wenzhu Li https://orcid.org/0000-0002-6560-9779
Muhammad Shoaib https://orcid.org/0000-0001-8695-1840
Amjad Islam Aqib https://orcid.org/0000-0001-7618-3948
Ruofeng Shang https://orcid.org/0000-0003-2814-0761
Zhen Yang https://orcid.org/0000-0003-1495-5206
Wanxia Pu https://orcid.org/0000-0002-4607-5855