Microbiological safety and antibiotic resistance risks at a sustainable farm under large-scale open-air composting and composting toilet systems

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ABSTRACT:

This study evaluated the microbial safety and antibiotic resistance risks of a sustainable ecological farm under large-scale open-air composting (OC) and green composting toilet systems (CT). Samples of livestock manure, compost, soil, vegetables, and rainwater were analysed to determine the best treatment of wastes and risk assessment of land application. Results showed that pathogenic bacteria (PB) in livestock manure was significantly greater than that in the surrounding topsoil, while the distribution of bacteria resistant to amoxicillin (AMX), tetracycline (TC), and amoxicillin-tetracycline (AMX- TC) was the opposite through long-term resistance selection pressure. *E. coli* and *Enterococcus* were the dominant pathogens in feces and surrounding soil, respectively, and AMX-resistant bacteria dominated soil, compost, and vegetable samples. Overall, while OC may significantly increase antibiotic resistance and effectively remove fecal PB, CT offers faster consumption with greater antibiotic resistant bacteria (ARB) removal but more PB. Moreover, PB and ARB were concentrated in mature compost, soil in planting areas, vegetables, and rainwater. In farm soil and vegetables, AMX-resistant and AMX-TC-resistant bacterial communities displayed similar composition. These findings may explain the main pathways of PB transmission, migration and accumulation of ARB in farms, and the potential risks to human health through the food chain.

KEYWORDS: pathogenic bacteria, antibiotic-resistant bacteria, aerobic composting, land application, risk assessment

1. Introduction

In recent years, the problem of antibiotic resistance [1-3] has become a major public health crisis [4-9] and has triggered a sharp increase in medical costs and mortality worldwide [2-5, 8-16]. Such resistance is induced by the abuse of antibiotics in the prevention and treatment of human and animal diseases and growth promotion in livestock [12, 13, 15, 17-23]. It is estimated that the annual global use of antibiotics is between 100,000 and 200,000 tons [18], of which the consumption of antibiotics increased by about 69% from 2000 to 2015, an increase of more than 4% per year [19]. In the United States, the annual use of antimicrobial compounds is 16,000 tons, 70% of which is used for non-therapeutic purposes. In China, the total antibiotic production in 2013 was 248,000 tons, of which 84,240 tons were used for veterinary antibiotics [24]. In Europe, veterinary antibiotics account for one-third of total use. According to the assessment of the antimicrobial resistance (AMR) crisis, the number of deaths caused by AMR in the world could be as high as 300 million, and the economic loss could reach USD 60-100 trillion in the next 35 years [15]. In 2014, a World Health Organization (WHO) report revealed that antimicrobial resistance has been observed in all WHO-defined regions of the world [8] and estimated that at least 50,000 people in Europe and US may die from drug-resistant bacterial infections every year [2]. In 2015, WHO regarded antibiotic resistance as one of the major global public health threats on a worldwide scale [3]. With the joint support of the UK Government and Wellcome Trust, the Review on AMR produced its final report and recommendations in 2016 to understand and propose solutions to the problem of drug-resistant infections from an economic and social perspective. In 2017, the Ministry of Agriculture of the People's Republic of China launched the National Action Plan to Combat Bacterial Resistance from Animal Sources (2017-2020) [13]. Similarly, in 2019, the UK launched the Five-Year National Action Plan on Antimicrobial Resistance (2019-2024) to reduce the use of antibiotics [2].

Due to long-term exposure to the selection pressure of host antimicrobial treatment, intestinal symbiotic bacteria (such as *Escherichia coli* and *Enterococci*) in animals and humans are considered good indicators of the overall level of antibiotic resistance [1, 25]. The drug resistance genes of these bacteria can also be transferred to various pathogenic bacteria (PB) [25]. *Escherichia coli*, a pathogen that causes diarrhoea and other intestinal diseases, is widely used as an indicator of microbial quality in water and food. Some strains of *E. coli* exhibit highly toxic properties and can be deadly, thus their detection and removal are pertinent [26, 27]. In Bangladesh, besides rotavirus, pathogenic *E. coli* is the second leading cause of diarrhoea [26]. In addition, as the main intestinal symbiotic bacteria, multi-drug resistant *E. coli* may also cause common and severe bacterial infections, such as urinary tract infections and sepsis [16], which has attracted widespread attention in countries around the world. Since 2014, the European Commission has issued an important resolution requiring EU member states to monitor the drug resistance of commensal *E. coli* [1]. Previous studies have repeatedly confirmed the isolation of pathogenic and antibiotic-resistant *E. coli* from environmental samples [26]. For instance, β-lactamase-producing *E. coli* was also isolated and identified from fish in the Mekong Delta [6].

In 2011, due to *Shiga Toxin-Producing E. coli O104: H4* in contaminated sprouts, a huge epidemic involving 3,842 cases and 53 deaths was triggered in Germany [28]. *Enterococcus* spp., another major naturally occurring symbiotic bacterium in humans and animals, is often used as an indicator of antibiotic resistance in food animals. Because of its intrinsic and acquired resistance to multiple antibiotics, *Enterococcus* has also become one of the main causes of nosocomial infections [10]. Recently, vancomycin-resistant enterococci were listed among the crucial antibiotic-resistant bacteria (ARB) in the "global priority list of antibiotic-resistant bacteria for the research and development of new antibiotics" by WHO [10]. Notably, although zoonotic disease transmission is rare, resistant enterococcal strains can be used as reservoirs of antibiotic-resistant genes (ARGs) for other pathogens in the gut [10, 29]. The increasing resistance of intestinal pathogens to existing antibiotics has attracted worldwide attention [26, 30] as it may seriously hinder the treatment of infectious diseases [26]. Specifically, pathogens that carry single / multiple ARGs [31] may infect humans through drinking water or the food chain, posing a serious threat to the safety of global public health [20, 32]. It is estimated that the annual deaths due to ARB in the US and the EU are approximately 23,000 and 25,000, respectively [30].

With the continuous pursuit of ecological environmental protection and organic food intake [11], use of human and animal waste in composting and subsequent land application has become a routine procedure [26] which will enrich antibiotic resistance [4, 32, 33]. However, due to the abuse of antibiotics and their incomplete metabolism [18, 24], abundant residues of antibiotics, ARB, ARGs and mobile genetic elements (MGEs) in human and animal feces [4, 22, 23, 32] are consistently released into the environment, thus seriously threatening the ecological environment and human health [4, 18, 26, 32, 33]. In recent years, aerobic composting has been widely used as an effective bioremediation technology [18, 34, 35] in utilising manure as a harmless source for soil improvement [12, 14, 22, 28, 34]. Yet, numerous studies have reported significant differences in the removal effect of different antibiotics and resistance to them, which may further enhance the migration and spread of AMR [12, 18, 22, 28]. In addition, to better ensure food security and human health, the number of human pathogens in manure compost should be reduced to avoid pathogen contamination of crops [28]. Studies have shown that antibiotics, animal-derived bacteria, and their ARGs remain in manure and compost is introduced into the receiving soil with land application, which gradually migrate and spread into deeper soil layers and/or groundwater [4, 11, 17, 23]. This transfer subsequently influences the overall microbial community structure and soil activity, inducing the formation and development of antibiotic resistance in the bacterial community through selection pressure [7, 11, 22, 26, 30, 32, 36], prompting the soil to become a reservoir for ARBs [4, 7, 30]. Furthermore, ARBs and their genomes from soil or irrigation water can enter the food chain through contaminated plant-derived foods, especially produce that is typically eaten raw, posing a serious threat to animal and human health [5, 11, 21, 23, 26, 28, 30]. It has been reported that plants can absorb antibiotics through roots and accumulate sub-inhibitory concentrations of various antibiotics in different parts of the plant. Since the fitness cost of antibiotic resistance has been shown to be

low to the bacterial cell [30], resistant strains in microbial communities in plant-derived foods will be significantly superior to sensitive strains. Compared to the conventional production mode without manure compost, the absolute abundance of ARGs is higher in organically produced plants [4, 11, 23, 28]. In addition, the close association between ARGs and MGEs has led to the potential migration and transformation of antibiotic resistance between soil, plants, and zoonotic pathogens, which has significantly increased the potential risks to the ecological environment and human health [20, 30]. Therefore, research on antibiotic resistance of manure compost, the soil environment, water resources, and food has become a hot topic in recent years. However, only a few studies have simultaneously analyzed the composting treatment mode of two manure-derived wastes (human / animal) and comprehensively assessed the microbial safety and antibiotic resistance risks in the overall environment of a sustainable ecological farm under their land application.

In this study, a sustainable ecological farm, closest to the urban area of London, with the typical human and animal manure composting systems was selected. Various environmental samples from the farm included the main types of livestock manures and topsoil samples beneath them, composting samples of human or animal manure of different maturity, several edible vegetables grown in fertilized soil and topsoil samples near sampled plants, and rainwater collected for irrigation and uncontaminated soil without fertilization within two years. After samples were collected, the effects and potential risks of the large-scale open-air aerobic composting and the green composting toilet systems on the microbial safety and antibiotic resistance risks in the farm environment were comprehensively and systematically analysed. The investigation was aimed to thoroughly identify the existing security risks and health threats to the sustainable ecological management model posed by human / animal manure-derived waste treatments in their land application and organic farm cultivation.

2. Materials and methods

2.1 Farm composting system and sample collection

The farm targeted in this study was an urban farm in central London and is in one of the most densely populated wards of Tower Hamlets (as shown in Figure S1). This sustainable ecological farm breeds more than a dozen animals, including chickens, pigs, donkeys, goats, and sheep, and produces a variety of vegetables and fruits grown in open air and greenhouses for visitors and nearby residents. In addition, the farm's daily waste (livestock manure, food waste, damaged fruits and vegetables, etc.) is used as the main raw materials for the large-scale open-air composting (OC) process of the farm as a reasonable recycling and resource utilization. This process mainly depends on two large-scale open-air composting heaps and four small-scale open-air composting bays (as shown in Figures S2 and S3), which undergo static composting and heaps-to-bays transfer at different time stages to achieve the gradual maturation of organic fertilizers and sustainable development of the farm's ecosystem. The detailed process and different time stages (stages I-IV) of OC were shown in Figure S4. It can be seen from

Figure S4 that the initial stage of OC test (about 0-3 months, stage I) mainly includes two processes: continuous feeding and static composting, in which the initial material of OC will continue to increase with the continuous production of the farm's daily waste. With the continuous increase in composting time and materials, OC will enter the next stage (about 3-6 months, stage II) when the heap's capacity is basically saturated (the heap depth of about 1.6-1.9 m), that is, the closed static composting stage without any feeding. Subsequently, the composting materials of stage II are transferred to the composting bays (the average depth of about 1.2-1.5 m) for a three-month secondary static composting (stage III), and finally the mature compost (about 9-12 months, stage IV) is applied or sold.

The farm is surrounded by schools, parks, and residential areas and has established close cooperation with many companies and universities to ensure its long-term and stable visitor flow. Since 2017, University College London has collaborated with the farm to intall and operate a vacuum composting toilet (CT) system (Envirolet® FlushSmart VF 750 AC Vacuum Flush System, Sancor Industries Ltd, Canada), which achieves effective water conservation (0.2 L/flush) while recycling the nutrients contained in human waste. This CT system (as shown in Figure S5) connects the vacuum micro-flushing toilet through pipes with two composting units (as shown in Figure S5(C)) placed in parallel (Details displayed on company website: https://envirolet.com/collections/envirolet-flushsmart-vf-composting-toilet-systems/products/envirolet-flushsmart-vf-750-ac-ac-electric-double-tank). During the test period, the CT system will be continuously opened during the normal operation time of the farm and fresh human waste will be added irregularly according to the actual use of users. After each flush, the newly added human-waste is evenly distributed into the two composting units and fully mixed with the previous mixtures, the daily farm waste (coffee grounds and sawdust, etc.) supplemented regularly, and with a microbial accelerant to assist the composting. Meanwhile, the Envirolet®/SG automatic six-way aeration™ system and the venting system (as shown in Figure S5(D)) are used to complete the automatic aeration and ventilation within the composting unit to facilitate the smooth maturation of the compost. The detailed working processes of Envirolet®/SG composting toilet and aeration systems are listed in Table S1 of the Supplementary material.

The compost maturity of the farm compost is mainly determined based on external characteristics, such as color and odor, in which mature fertilizer is used for planting fertilizer needs and the rest is packaged and sold to the citizens of London. It can be seen that the farm OC must effectively balance and solve the sustainable development relationship between the daily waste production and the actual composting capacity while processing a large number of complex and difficult-to-degrade raw materials generated continuously for a long time. In addition, open-air and static composting without any external assistance (no ventilation, oxygen supply and microbial accelerant addition) will also be limited by external objective environmental conditions (such as weather and seasons), as well as the real situation of long-term storage of rotten compost caused by the actual supply and demand of the farm (as shown in Stages III-IV of Figure S4). Therefore, the OC with large scale and

continuous addition of fresh materials may take longer to mature than CT. The length of time that the OC' compost matures for is 9-12 months, with some variation depending on the supply of waste and season. To comprehensively and systematically evaluate the effects and potential risks of the OC and green CT system on microbial safety and antibiotic resistance in the farm environment, 21 kinds of environmental and compost samples were analysed during the farm ecosystem cycle (Table 1). Among them, the unfertilized 2-year soil samples (US) collected within the scope of sustainable ecological farm and close to the planting area and the breeding area with similar basic conditions were used as the blank control group, so as to better analyze and further reveal the impact of manure / compost application on microbiological safety and antibiotic resistance risks in farm soil environment. Moreover, the planting areas of the three vegetables selected were all soil areas with continuous fertilization for 2 years and fixed planting varieties (i.e., single fertilization method) to carry out comparative analysis with US. Furthermore, the two composting systems that were emptied and in good condition before the test will always be in the daily use state under the routine operation of the farm before sampling to truly reflect and accurately evaluate the potential risks of the OC and CT compost products ultimately used for land application. Except for the vegetables, all samples were homogeneous, mixed after random multi-point sampling, and transferred to the laboratory for timely processing on the same day.

Table 1. Sample information sheet for farm sampling

Number	Sample information	Sample abbreviation	Sample source	Number	Sample information	Sample abbreviation	Sample source
1	Donkey manure	DM	Breeding areas	12	Toilet compost for 2 months	TC	Composting toilet systems
2	Donkey manure covered topsoil*	DS		13	Initial compost for 3-6 months	IC	Large-scale open-air composting
3	Goat manure	GM		14	Mature compost for 9-12 months	MC	
4	Goat manure covered topsoil	GS		15	Topsoil around tomatoes	TS	Planting areas with continuous fertilization for 2 years
5	Sheep manure	SM		16	Topsoil around saltbush	SAS	
6	Sheep manure covered topsoil	SS		17	Topsoil around radishes	RS	
7	Chicken manure	CM		18	Tomatoes	TO	
8	Chicken manure covered topsoil	CS		19	Saltbush	SA	
9	Pig manure	PM		20	Radishes	RA	
10	Pig manure covered topsoil	PS		21	Collected rainwater for plant irrigation	CR	Farm rainwater collection device

11	Uncontaminated topsoil	US	Farm soil without fertilization in two years	

* The topsoil collected in this study refers to soil samples at a depth of 10 cm covered by manure / compost around the sampling site.

2.2 Chemicals and reagents

Amoxicillin (AMX, ⩾90%) and tetracycline hydrochloride (TC, ⩾90%) were purchased from Sigma-Aldrich, USA. Eosin methylene blue agar (Oxoid, UK), bile esculin agar (Honeywell Fluka ™, US), and R2A agar were used for the selective culture of E. coli, Enterococcus, and environmental bacteria, respectively.

2.3 Biological index measurement and analysis methods

2.3.1 Viable counts of PB and antibiotic-resistant bacteria

The viable counts of PB were counted on the eosin methylene blue agar and bile esculin agar without antibiotic addition. According to the maximum value of the Minimum Inhibition Concentration (MIC) of bacteria listed in Clinical and Laboratory Standards Institute [37], AMX-resistant bacteria, TC-resistant bacteria and AMX-TC-resistant bacteria were counted on R2A medium supplemented with 32 mg/L AMX, 16 mg/L TC and the mixture of 32 mg/L AMX and 16 mg/L TC, respectively; the R2A medium without antibiotics was used as a control group to count the total culturable bacteria (TCB). Vegetable samples washed with water should be separated in the biosafety cabinet in advance and the crushed edible portion was used as the vegetable samples for subsequent cultivation. First, all 1.0 ml aqueous samples or 1.0 g wet solid samples collected in this study were fully suspended in 9ml sterile physiological saline (PBS, 0.85%) by vortex; secondly, the PBS suspensions of the above 21 samples were prepared into serial gradient dilutions (10^{-1} - 10^{-7}); finally, 50 μL of diluted samples were respectively spread onto the six types of plates, of which two PB plates need to be cultured at 35 ℃ for 24 h, four R2A plates with and without antibiotics were cultured at 25 ℃ for 5 days. The colony formation unit (CFU) per mL or g was calculated by formula (1) [38, 39]. All measurements were made in duplicate.

$$\mathrm{CFU}\left(\frac{1}{\mathrm{mL}}\right)=\frac{colony\ count}{sample\ volume(mL)\times dilution rate} \quad (1)$$

2.3.2 16S rRNA gene sequencing and identification of isolates

To identify AMX-resistant, TC-resistant, and AMX-TC multi-resistant bacteria, 30 dominant single colony morphologies that repeatedly appeared in most test samples were randomly selected from three plates with significant bacterial resistance as the main targets for isolation and identification. The operation procedures of colony PCR were performed according to existing literature [39], and details of the

specific conditions and parameters are provided in Supplementary Contents S1. PCR amplification products were analysed by 1.5% agarose gel electrophoresis (Analytik Jena, Germany). The 16S rRNA gene PCR products of each colony were sequenced by Source BioScience Limited (London). After sequencing, Advanced BLAST search program on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare the 16S rRNA gene sequence with other sequences in the GenBank database and ultimately obtain the identification results of each strain.

2.4 Physicochemical index measurement and analysis methods

The moisture content of solid samples (other than vegetable samples) was determined according to the TMECC standard method. After screening (≤3.5 mm), solid waste was subjected to leaching, centrifugation, and filtration through a 0.45-μm membrane according to British Standard BS EN 12457-1: 2002. Then, a laboratory pH-conductivity meter (Mettler Toledo, Switzerland), total organic carbon analyzer (Shimadzu TOC-L, Japan), and ion chromatograph (Dionex ICS 1100, US) were used to measure pH, electrical conductivity (EC), total organic carbon (TOC), and main anions, respectively.

2.5 Statistical analysis

Data statistical analyses were obtained using Excel 2016 (Microsoft, USA) and OriginPro 8.5 SR1 (OriginLab Corp., USA) software. Principal component analysis (PCA) and redundancy analysis (RDA) were performed using CANOCO 5.0.

3. Results and discussion

3.1 Characterisation analysis of the basic physicochemical parameters of the samples

Generally, part of the water in the compost will be evaporated or consumed with the high temperature and microbial decomposition during composting [40], but it can be seen from Figure 1(a) that the moisture content in the OC process slightly increased. This may be due to the complex raw materials (livestock manure, food waste, damaged fruits and vegetables, etc.) and static non-mixed mode of the farm OC, which resulted in the materials with high moisture content (fruits, vegetables, and food waste) that were not completely degraded in the initial stage (IC), but to a certain extent, they increased the moisture content of uniform MC in the maturity stage, thereby making up for the water loss during composting. The moisture content of whole soil in the planting area was higher than that in the breeding area, which may be due to the external water replenishment during artificial irrigation and the water-holding effect of plant roots. Compared to the IC, the TOC of toilet compost and MC were significantly reduced, consistent with the results reported by K. Sharma et al. [41], which may be due to the increasing carbon mineralization and humus richness as well as the degree of composting maturity. The TOC of the soil in the planting area was slightly lower than that in the breeding area, most likely because the soil in the breeding area was continuously polluted by animal wastes [42] and soil mineralization in the planting area [41].

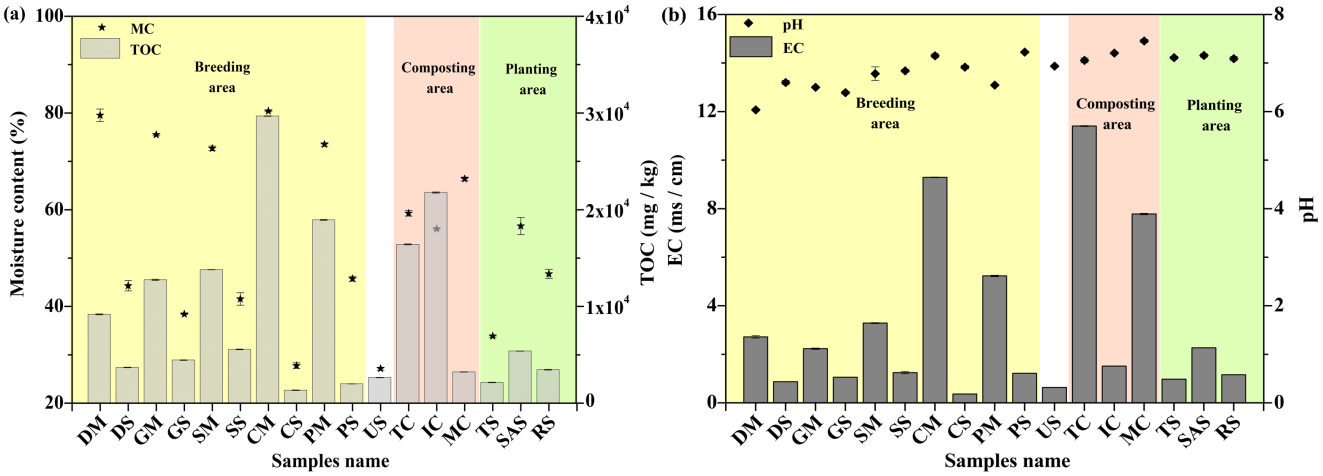

Figure 1. Basic physicochemical parameters of farm samples: (a) moisture content and TOC; (b) EC and pH.

Figure 1(b) shows that fecal EC in the breeding areas was significantly higher than soil EC, reflecting the potential inhibitory and toxic effects of livestock feces on plant growth [43, 44]. Compared with the IC, the EC of MC increased significantly, especially for the toilet compost, which may be caused by the concentration effect of compost materials and formation of inorganic salts [43]. Previous studies have shown that EC is a direct indicator of the salinity and maturity of compost [43]. As shown in the figure, the different pH values of the three compost samples may be contributed to a large amount of ammonium nitrogen produced by ammoniation during aerobic composting, leading to an increase in pH [24, 40, 45, 46].

3.2 Evaluation of microbiological safety and antibiotic resistance risk in the farm environment

3.2.1 Evaluation of microbiological safety in the farm environment

It can be seen from Figure 2(a) that *E. coli* in livestock manure in the breeding areas was significantly higher than that of soil *E. coli*. Specific differences were noted between the chicken and pig groups (CM/CS, PM/PS), where *E. coli* pollution in CM and its surrounding surface soil (CS) was the greatest in the breeding areas. The efficient removal of PB and ARB from common manure materials for aerobic composting [40, 46], such as CM and PM, is closely related to the safety of organic plant food for humans, fully reflecting the necessity and critical nature of pre-treating manure before land application [5, 7, 11, 20, 21]. Compared with the feces in the breeding areas, the amount of *E. coli* in compost samples was significantly reduced after the OC treatment. Moreover, the *E. coli* quantity decreased as the composting time increased, which indicates that the traditional aerobic composting treatment on PB in feces can effectively remove bacteria. However, the amount of *E. coli* in the TC was significantly higher than that of most livestock manure and traditional OC compost, indicating the high levels of *E. coli* in human waste. This may be due to the continuous feeding mode of the composting toilet, resulting in the final sampled toilet compost including all compost with different feeding time and maturity, in which the fresh unfermented or short composting time human wastes are likely not to experience

the sanitization stage and not fully mature before sampling, thus leading to a higher content of *E. coli* in toilet compost. This finding also suggests that two-month-old toilet compost is not safe for land application. As shown in Fig. 2(a), the surface soil around different vegetables contained different amounts of *E. coli*, which was probably caused by different factors such as fertilization strategy, plant nutrient demand and its absorption rate, soil specific environment and so on. For rapid plant maturity and better harvest, the compost application method will also vary with the different characteristics of various plant growth and output parts. The fertilization method of TO and SA was mainly to spread compost around the plant and cover the topsoil, while RA was a method of applying compost deeply to the root of the plant and mixing it with the soil. As a result, TS and SAS may be more affected by compost contamination, and their samples may inevitably contain mixed composting particles. Moreover, the CR for irrigation also possessed a certain amount of *E. coli*. In addition, the amount of soil *E. coli* in the planting areas was significantly higher than that in the breeding areas, which may be due to the effect of long-term continuous use of organic fertilizers and polluted CR on changing the community structure of soil microorganisms. This change significantly increases the accumulation of PB in the soil and will further threaten the safety of the farm, its surrounding environment, and human health [4, 7, 22, 26, 32].

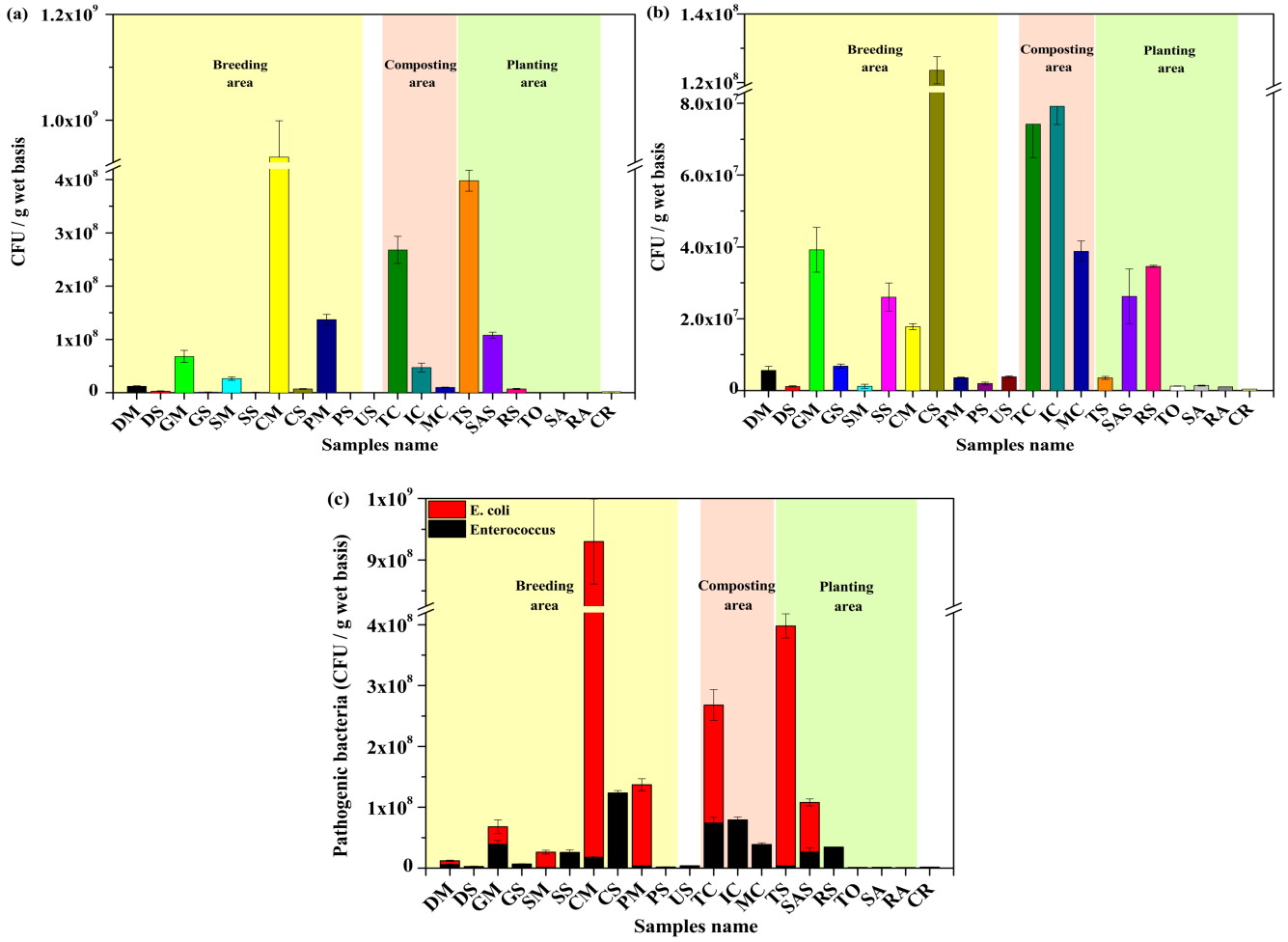

Figure 2. Counting results of PB quantity in farm samples: (a) *E. coli*, (b) *Enterococcus*, and (c) stack column of both.

As shown in Figure 2(b), except for sheep (SM / SS) and chicken (CM / CS) groups, the number of *Enterococcus* in other livestock manures in the breeding area was higher than that in the surrounding topsoil. However, the amount of *Enterococcus* detected in CM was far less than that in the surface soil, which may be due to that CM with high moisture content (as shown in Figure 1(a)) was more likely to invade and accumulate in the topsoil layer. In addition, the environmental conditions of the chicken coup were more conducive to promoting the growth of *Enterococcus* in the soil. Therefore, a certain amount of *Enterococcus* remaining in CM will gradually migrate, diffuse and accumulate into the soil with the long-term and repeated land cover, prompting the CS to become a reservoir [4, 7, 30] for *Enterococcus* through positive selection pressure. He et al. [4] also reported that antibiotic residues and animal-derived bacteria can persist in the soil after manure application, and even transfer to groundwater after several years. Moreover, the US contained higher levels of *Enterococcus* than the topsoil in some breeding areas, which may be contaminated by feces of various free-range animals (including chickens, goats, sheep and donkeys) during the daily operation of the farm, especially affected by long-term free-moving chickens. Comparing the samples from the breeding area, it can be seen that the number of *Enterococcus* in the compost samples was significantly higher. As the composting maturation process continued, *Enterococcus* in the OC samples decreased significantly, further verifying that the composting process has a removal effect on *E. coli*, *Enterococcus*, and other PB. As can be seen Figure 2(b), *Enterococcus* in the topsoil in the planting area was generally higher than those in the soil samples of the breeding area and the blank control group (US), and a large amount of *Enterococcus* was also enriched in the three common edible vegetables and contaminated CR to a certain extent. This finding indicates that the long-term application of contaminated organic fertilizer and CR can change the soil microbial community structure and promote the accumulation and transmission of PB communities in the food chain, thereby posing a potential threat to human health [5, 23, 26, 28, 30].

Figure 2(c) also shows that the PB number in feces in the breeding areas was significantly higher than that in the surrounding surface soil, while the feces of chicken and pig groups and their surrounding topsoil were heavily contaminated by PB. In addition, *E. coli* dominated the PB in the manure of the breeding areas, while *Enterococcus* was the dominant PB in the surface soil of the breeding areas. Both *E. coli* and *Enterococcus* were the dominant PB in the CT and traditional OC samples. The PB contamination in the toilet compost was relatively high, indicating that the CT' composting cycle corresponding to this sampling point was not effective enough to eliminate the contamination of PB completely in human waste. It is reported that the maximum value of *Enterococci spp.* or *E. coli* in one gram of compost should not exceed 1000 CFU (3.0 log10) to meet the requirements of compost sanitization [34]. In Ontario Canada, CP1 and CP2 are divided according to the strictness degree of biosolids treatment. CP1 strictly requires that the abundance of *E. coli* in biosolids be reduced to a maximum of 1000 CFU per 100 ml, which is equivalent to Class A biosolids in US parlance; Biosolids that have been insufficiently treated to meet these standards are designated CP2 or Class B in US, which must also meet the *E. coli* less

than 2,000,000 CFU / g of total solids dry weight standard [5]. It can be concluded that the content of *E. coli* (9,900,000 CFU / g and 268,000,000 CFU / g) and *Enterococcus* (38,800,000 CFU / g and 74,200,000 CFU / g) in the final samples of OC and CT were far beyond the above standards, which may potentially threaten the ecological environment and human health. It can be seen from Figure 2(c) that, the accumulated PB in TS was much higher than that in other planting area and the blank control group (US) without fertilization for 2 years. It may be affected by the different fertilization methods developed by the farm according to the growth needs of different plants and their respective yields. Among them, TO and RA need more fertilization and multiple topdressing due to the needs of ripening, fattening and increasing yield, while SA only needs a small amount of fertilizer to obtain its fresh leaves as salad ingredients. Considering the specific operation and location of compost application, the PB contamination of TS was likely to be more serious than samples from other planting areas. As shown in Figure 2(c), the soil in the planting area was relatively enriched with a large quantity of PB, among which E. coli accounted for the absolute advantage of the total PB in other surface soil samples except RS. In addition, the comprehensive analysis of Figure 2(a)-2(c) shows that the three common edible vegetables in the planting area also have a certain number of PB due to the long-term application of contaminated compost and CR and their growth in the soil with serious PB pollution. This finding reveals that these bacteria may enter the food chain through plant-derived food and threaten the health of animals and humans [23, 28]. Therefore, *E. coli* (7,100,000-398,000,000 CFU / g and 1,400-3,800 CFU / g) and *Enterococcus* (3,540,000-34,600,000 CFU / g and 1,040,000-1,386,000 CFU / g) with high abundance in the soil and vegetables in the planting area were likely to present long-term potential human health risks.

3.2.2 Risk assessment of antibiotic resistance in farm environment bacteria

As shown in Figure 3(a), with the exception of the pig group (PM/PS), the number of AMX-resistant bacteria in the soil of the breeding areas was greater than that in the feces. In Figure 3(d), it can be seen that the proportion of AMX-resistant bacteria (AMX/TCB) in the soil of the breeding areas was significantly higher than that of the fecal groups, especially the chicken, goat, and donkey groups. This may be due to the gradual migration and diffusion of the residual AMX and its ARB and ARGs from the manure into the covered surface soil and their long-term continuous accumulation. Meanwhile, AMX resistance in the soil significantly increased through positive selection pressure, which is even higher than the risk of antibiotic resistance in feces. The above results were also consistent with the previous studies [4, 7, 11, 17, 22, 23, 26, 30, 32, 36], and fully reflected the existing potential risks and safety threats of the migration and diffusion of antibiotic resistant determinants to deeper soil under the condition of long-term coverage of animal manure in breeding areas of the farm. As shown in Figure 3, the number and resistance ratio of AMX-resistant bacteria in OC samples were significantly higher than those in CT, and AMX resistance gradually increased with the continuous maturation of OC. This may be caused by the positive selection pressure during OC's long-term composting process,

which promoted the increasing and accumulation of antibiotic resistance determinants [18, 22]. Although the number of AMX-resistant bacteria in vegetables in the planting areas was significantly lower than that in the surrounding topsoil samples, the proportion of AMX resistance in vegetables was only slightly different from that in the topsoil samples. It can be seen from Figure 3(a) and Figure 3(d) that the AMX resistance of some vegetables and their surrounding soil in the planting area was generally higher than that of most samples in the breeding area and compost, especially the soil samples in the planting area were the most significant. M. Xu et al. (2019) reported that inactivation of antibiotic resistome in natural environments is difficult [47, 48] due to their mobility and persistence [22]. Therefore, the AMX-resistant bacteria accumulated continuously in soil and vegetables in planting area under the selective pressure of long-term fertilization in this study, which were as high as 27,400,000-212,000,000 CFU / g and 370,000-6,440,000 CFU / g respectively, were likely to be gradually exposed to human through the food chain, causing a serious threat to human health [5, 11, 28, 30].

Figure 3(b) displays that the number of TC-resistant bacteria in the feces of the breeding areas as a whole was more than that in the topsoil samples covered by them. However, the proportion of TC resistance (AMX / TCB) in other fecal samples, except donkey and sheep, was significantly lower than that in soil samples, especially in the pig group (PM/PS). This further verifies the migration and transmission path of antibiotic resistance from livestock feces to receiving soil. According to Figures 3(b) and 3(e), the TC resistance was significantly enhanced during traditional OC. Compared to the composting time and drug resistance level of CT, the long-term OC processes cannot effectively eliminate antibiotics but may become an antibiotic resistance reservoir, thereby increasing the accumulation and spread of environmental antibiotic resistance. In addition, the TC-resistant bacteria (9,700-1,880,000 CFU / g) and ratio (21.95-94.77 %) of TC/TCB in vegetables in the planting areas were generally higher than those in the surrounding surface soil (40,000-140,000 CFU / g; 0.16-25.25%). Moreover, TC resistance in bacterial species isolated from vegetables was significantly higher than that of breeding areas' soil and compost samples. Thus, it can be suggested that TC-resistant bacteria and their genomes in compost and soil may enter plants through the absorption by plant roots and form resistance selection pressure inside plants, thereby inducing the formation and development of antibiotic resistance in plant communities [28, 30].

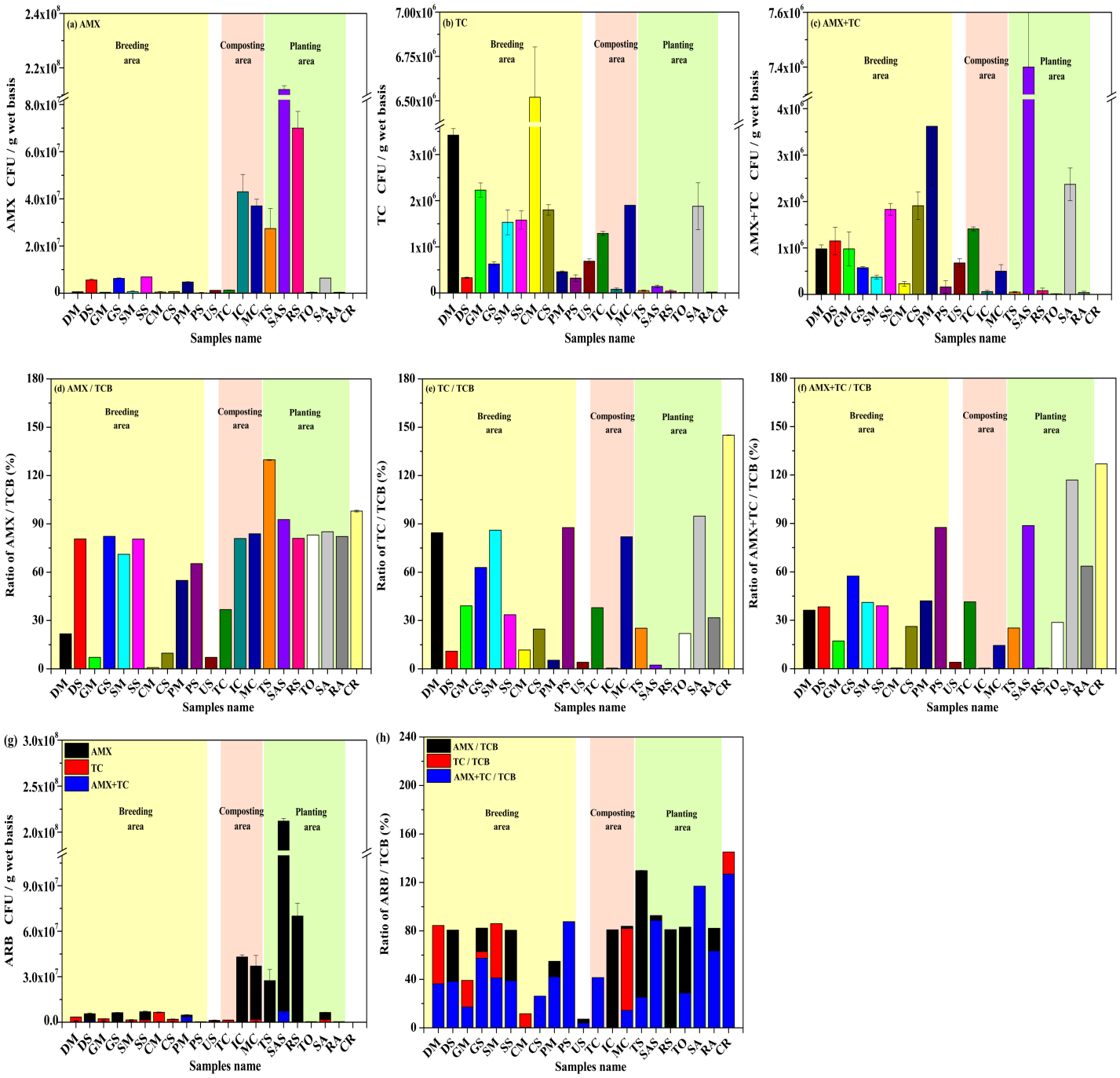

Figure 3. Results of the total counts of (a) AMX, (b) TC, and (c) AMX-TC resistant bacteria; the resistance proportions of (d) AMX / TCB, (e) TC / TCB, and (f) AMX-TC / TCB; and the cumulative histograms of both (g) ARBs and (h) resistance proportions.

As shown in Figure 3(c), the number of AMX-TC multi-drug-resistant bacteria in the feces of other breeding areas, except goat and pig groups (GM/GS, PM/PS), was lower than that of soil samples. In Figure 3(f), it can be observed that the overall proportion of fecal resistance in breeding areas was lower than that in the topsoil covered by them, which may be due to the long-term selection pressure of residual antibiotics in the manure, leading to acquisition and gradual enhancement of multi-drug resistance in the soil. Based on Figures 3(c) and 3(f), it can be proposed that OC treatment cannot effectively remove antibiotic resistance in feces, and the proportion

of AMX-TC multi-drug-resistant bacteria and their resistance will increase significantly with composting maturation. A comprehensive assessment of composting times and drug resistance levels showed that CT took a relatively short time and exhibited low levels of antibiotic resistance, which indicates that the potential risk of drug resistance was lower when applied to land. As shown in Figure 3, the overall resistance ratios of vegetables in the planting areas and the surrounding topsoil were slightly higher than that of the manure and topsoil samples in the breeding areas. The proportion of multi-drug resistance of raw vegetables was also significantly higher than that of the surrounding topsoil, objectively revealing the transmission, migration, and accumulation of antibiotic resistance via human and animal fecal-derived waste, compost, soil in planting areas, and plant-derived food, which are eventually ingested by humans [4, 5, 11]. In addition, recently unfertilized soil and irrigation rainwater all contained AMX, TC, and AMX-TC antibiotic resistance to a certain extent. In particular, rainwater collection displayed a significant proportion of drug resistance, which can be transferred to the soil and vegetables in the planting areas through daily irrigation, threatening the ecological environment and human health.

Figures 3(g) and 3(h) provide a comprehensive analysis of the distribution of antibiotic-resistant bacteria and their resistance ratios in all samples on the farm, which indicates that AMX resistance dominated in most samples, especially soil, compost, and vegetables. In addition, the level of drug resistance in the soil in the breeding areas was higher than that in the livestock manure. A comprehensive comparison of the antibiotic resistance levels in all samples collected on the farm showed that multiple single / multi- drug resistances were mainly concentrated in the sustainable production terminals of the farm. These terminals include mature compost, CR for irrigation, surface soil in planting areas, and raw vegetables, which provide direct pathways into human body through the food chain. The recently adopted Regulation (EU) 2019/6 requires that all risks related to AMR development must be taken into account for veterinary drug products [17], but so far there is no generally accepted method to assess the development or spread risk of AMR in the environment [49, 50]. This has been reported that it may be limited by the current knowledge about the minimum antibiotic concentration for ARB selection [17, 51], so the MIC of sensitive bacteria is usually used as alternative data to predict the non-selective concentration of aquatic systems [17, 50]. In conclusion, the three ARBs in the vegetables (9,700-6,440,000 CFU / g) and the surrounding topsoil (40,000-212,000,000 CFU / g) in the farm planting area investigated in this study had relatively high human exposure concentration, which were likely to pose potential threats of antibiotic resistance transmission and serious human health risks.

3.2.3 Isolation and identification of antibiotic resistant strains

As can be seen from Table S2, 20 antibiotic-resistant strains were identified in different samples of the farm. Samples in the breeding areas mainly contained *Sphingobacterium*, *Pseudomonas*, *Variovorax*, and *Stenotrophomonas*; the compost samples contained *Sphingobacterium*; and the planting areas mainly contained

Psychrobacter, *Pseudomonas*, *Stenotrophomonas*, and *Variovorax*. Moreover, samples from breeding and planting areas displayed greater similarities of drug-resistant strains. Previous studies have reported that *Sphingobacterium*, *Pseudomonas*, *Stenotrophomonas*, and *Chryseobacterium* are dominant isolates with high antibiotic resistance in soil samples [52]. Among them, ARB *Pseudomonas* is resistant to many antibiotic classes (e.g., β-lactams) in nature and can acquire resistance through horizontal gene transfer and mutation [28], which spreads to vegetables through contaminated soil. In addition, *Pseudomonas* possesses the ability to decompose pectin can promote rapid spoilage of vegetables [28, 53, 54] and is also the dominant pathogenic drug-resistant bacteria in aquatic ecosystems [55]. It has been reported that *Psychrobacter*, a group with high abundance in fertilized soil, is tolerant to salt and cold and carries *AmpC-β*-lactamase, which exists in large quantities in both Antarctic guano soil and winter fertilized soil [56]. This is also consistent with the results of TC resistant and AMX-TC resistant *Psychrobacter* strains isolated from SA with high salt content in the planting area of this study. *Stenotrophomonas*, the dominant strain of *Gamma-proteobacteria*, is the most abundant bacterial population in various resistant media, such as AMX, ciprofloxacin, and sulfamethoxazole, and is an important hospital pathogen resistant to solar radiation and at least three antibiotics [37]. Herein the AMX- and TC-resistant isolates of *Stenotrophomonas* were detected in fecal, soil, and vegetable samples from the farm breeding areas.

Table S2 lists more AMX and AMX-TC-resistant strains (*Sphingobacterium*, *Pseudomonas* and *Variovorax*) found in the farm samples, among which *Sphingobacterium* was an isolated strain that simultaneously grew on three different resistant petri dishes. It has been reported that *Sphingobacterium*, one of the most abundant bacteria in soil samples, is significantly related to the abundance of TC and β-lactam ARGs [57]. *Pseudomonas* and *Variovorax* are common indigenous flora in soil and water, both of which are associated with a variety of rare metabolic characteristics, including degradation of toxic and complex compounds [58]. Among them, *Variovorax* can complete a variety of catabolic pathways, has other characteristics related to the mediation of metal ions, organic sulphides, and other compounds [58], and exhibits resistance to multiple antibiotics [58, 59]. Numerous studies have confirmed the contribution of compost application to vegetable microorganisms, especially to multi-resistant strains [28].

According to the identification results in Table S2, Venn diagram S4 displays the antibiotic resistance and sample classification. As shown in Figure S6(a), the number of common strains of AMX-resistant and AMX-TC multi-resistance in the farm samples was relatively high. This may be because the resistance genomes of AMX and TC often coexist in the same MGEs [13, 60], or the dominant drug-resistant bacteria under AMX selection pressure are more likely to acquire or develop multi-drug resistance to AMX-TC. In Figure S6(b), relatively high numbers of the same resistant strains were detected in the farm soil and vegetable samples, suggesting a similar composition of resistant bacterial communities between the two. This finding further validates that antibiotic resistance in cultivated soil may play a crucial and

decisive role in bacterial composition and drug resistance levels in plant-derived foods.

3.3 The relationship and mechanism between farm environmental factors and important bacterial communities

3.3.1 Correlation analysis

As listed in Table S3, there was a highly significant positive correlation between physicochemical indicators, such as moisture content, TOC, and sulphate, as well as between some physicochemical indicators and TC-resistant bacteria at the 0.01 significance level (two sided). This analysis reflects a close relationship between the physicochemical indicators of the farm samples and that physicochemical indicators, such as TOC, nitrite, and nitrate, were subject to a highly significant positive correlation with TC-resistant bacteria, *E. coli* and *Enterococcus*. This correlation may be attributed to the metabolism and other major life activities of environmental microorganisms, while the important physicochemical indicators may also significantly affect the occurrence, abundance and distribution of drug resistance [42]. In addition, the highly significant positive correlation between TCB and TC-resistant bacteria may be due to the fact that most TC-resistant bacteria were the dominant species of TCB and, thus, may change significantly with the increase or decrease of TCB. Furthermore, the high correlation between AMX and AMX-TC resistant bacteria was consistent with the results obtained in the Venn diagram (Figure S6(a)), fully demonstrating the close relationship between AMX and AMX-TC resistance. These results further reveal that the presence or absence of AMX resistance may significantly alter the emergence and development of AMX-TC multi-drug resistance.

According to Table S3, there was a significant positive correlation between moisture content, EC and TC resistant bacteria and between bromide, TCB, and TC resistant bacteria. In comparison, a significant negative correlation was noted between pH and phosphate. L. Tan et al. (2019) reported a significant correlation between antibiotic resistance and EC [61]. Thus, the proliferation and development of TC-resistant bacteria and TCB are closely related to important physicochemical parameters, such as moisture content, EC, bromide, and sulfate in the living environment. Such relation may further explain the internal relationship of mutual influence and mutual balance between microorganisms and their environment conditions.

3.3.2 PCA

According to the PCA, PC1 and PC2 accounted for 79.56% of the total variation (Figure 4). Figure 4 shows that the PB and ARB in the farm samples were clearly divided into three regions (manure in the breeding areas and its compost, soil, and vegetables) according to the different sources of the samples. This diversion directly reveals the main migration, diffusion, distribution, and accumulation pathways of the PB and ARB community in the farm environmental ecosystem, which is consistent with previous studies [62].

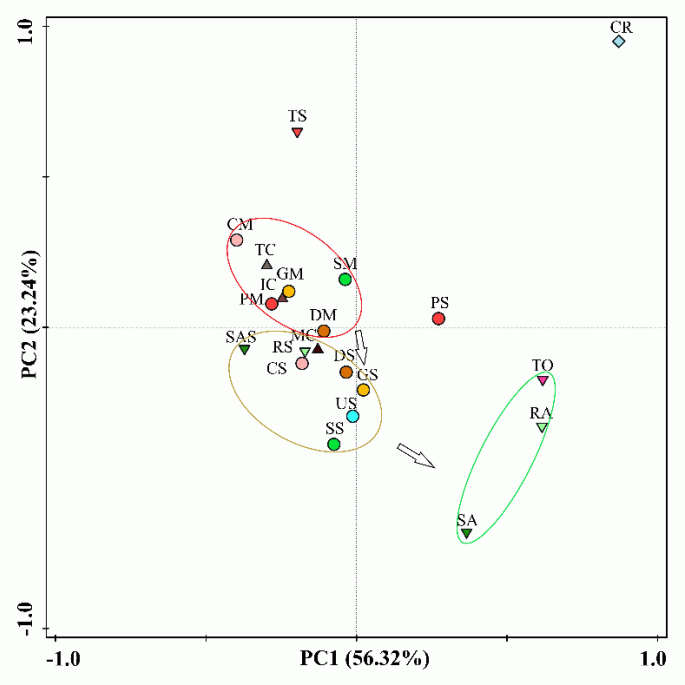

Figure 4. PCA of the main bacterial communities (pathogenic and antibiotic-resistant bacteria) in the farm samples.

As shown in Figure 4, the community structure of the main PB and ARB in the manure, toilet compost, and IC was similar. It may be due to the main PB, ARB, and their ARGs in human / livestock manure continuously diffused and accumulated in the compost environments under the long-term selection pressure of multiple antibiotic resistance in the composting process of the farm [12, 18, 22, 28]. With the composting maturation process and the long-term land application of compost, the differences in microbial resistance among the breeding soil under long-term manure coverage, MC, and planting soil gradually decreased. This may be caused by the introduction of PB and ARB residues in animal manure / compost into the receiving soil through long-term coverage or fertilization [4, 11, 17, 23,]. Subsequently, through positive selection pressure [7, 22, 26, 30, 32, 36], the overall microbial community structure and the development of PB and antibiotics resistance in the soil are affected, ultimately making the soil a reservoir for PB and ARB [4, 7, 30]. The three vegetables in the planting areas gradually grew and accumulated a certain amount of PB and ARB in the contaminated soil with long-term and repeated fertilizer land application. It can be seen from Figure 2-3 that the final MC used for plant fertilization and the topsoil samples in the planting area still contained more PB and ARB, and these PB and ARB are likely to continue to spread and accumulate in the vegetables through the absorption of plant roots [30], thus threatening human health through the food chain [5, 11, 21, 23, 26, 31]. Compared to the community composition gap between feces, compost, and soil samples, the differences were greater between the main bacterial community composition and structure of vegetables and other samples.

3.3.3 RDA

In this study, the RDA was used to better reveal the dynamic correlation between the bacterial community in the farm environment and environmental factors. The important environmental bacteria and their antibiotic resistance ratios as well as the basic physicochemical indicators of the farm were evaluated, which could explain 85.69% of the total variation of species.

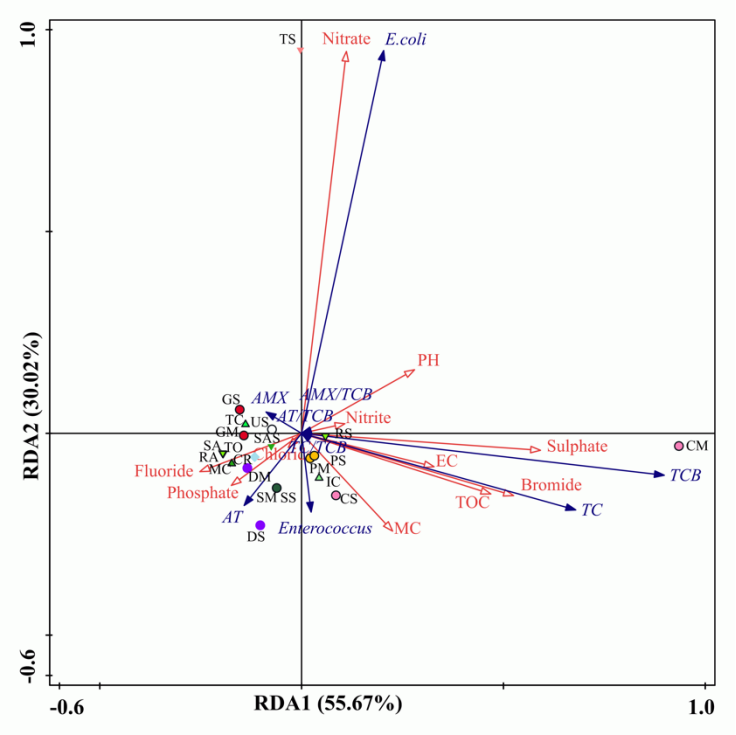

Figure 5. RDA of the relationships between the main bacterial communities (blue arrows) and environmental factors (red arrows): AT is the abbreviation of AMX-TC.

As shown in Figure 5, the overall distribution of farm environment samples was relatively compact. MC, CR, and vegetables were particularly concentrated, which directly reflected that there was little difference in the community composition of ARB and PB in the MC, CR, and plant-derived food. It may be due to MC and CR with a certain degree of PB and ARB contamination were likely to migrate, diffuse and accumulate into the soil and plant-derived food in the planting area with its long-term and repeated land application, thus significantly altering the bacterial community structure and drug resistance of vegetables and narrowing the composition gap between MC, CR and vegetables. This further revealed that the migration and stability of antibiotic resistance may be due to organic fertilizer application and crop cultivation on the farm, indicating that long-term fertilization and irrigation operations may have a significant impact on bacterial community structure and drug resistance of farm crops. The above results were also consistent with the previous research reports [5, 7, 11, 21-23, 26, 30-32, 36], and fully reflected the existing potential risks and safety threats of the sustainable ecological farm under the aerobic composting treatments of human and animal wastes. Figure 5 shows a significant correlation between most of the physicochemical indicators of the farm samples that are significantly related to *E. coli*, TCB, and TC-resistant

bacteria and may be related to the internal characteristics of the environmental samples and bacterial metabolism. In addition, there was a close correlation between TCB and TC-resistant bacteria and especially between AMX, AMX-TC and various environmental points in the farm. These correlations demonstrate the inherent close relationship between the occurrence and development of multiple antibiotic resistances, as well as the potential risks and health threats of existing ARBs, such as AMX, TC, and AMX-TC, in various environmental samples on the farm.

4. Conclusions

This study comprehensively investigated and truly evaluated the microbial safety (*E. coli*, *Enterococcus*) and potential antibiotic resistance risks (AMX, TC, AMX-TC) of various environmental samples and compost products of the eco-friendly farm under the two composting treatments (OC, CT), to completely understand and further reveal the existing security risks and health threats to the sustainable ecological management mode posed by human / animal manure-derived waste treatments in their land application and organic farm cultivation. The results showed that the PB number in feces in the breeding areas was significantly higher than that in the surrounding topsoil, while the distribution of bacteria resistant to AMX, TC, and AMX-TC was the opposite through the positive long-term resistance selection pressure. *E. coli* and *Enterococcus* were the dominant PB in animal manure and surface soil of the breeding areas, respectively, and AMX-resistant bacteria dominated soil, compost, and vegetable samples. Comparatively, OC increased antibiotic resistance but effectively removed PB in farm waste, while CT was quicker, had greater ARB removal but more PB. The final samples of CT with high levels of *E. coli* (268,000,000 CFU / g) and *Enterococcus* (74,200,000 CFU / g) may potentially threaten the ecological environment and human health, which need further sterilization treatment. Moreover, PB and ARB were mainly concentrated in the farm's sustainable production terminals, such as mature compost, CR, soil in planting areas and raw vegetables, which can directly affect the migration and accumulation of PB and ARB from farms into humans through the food chain and then seriously threaten human health. Therefore, the two PBs and three ARBs in the vegetables (1,400-1,386,000 CFU / g and 9,700-6,440,000 CFU / g) and their surrounding topsoil (3,540,000-398,000,000 CFU / g and 40,000-212,000,000 CFU / g) had relatively high human exposure concentration, which were likely to pose potential threats of antibiotic resistance spread and serious human health risks. To ensure microbial safety and limit antibiotic resistance risks, the process conditions for the two composting treatments should be further optimized on this basis, such as appropriate extension of the CT oxygen supply and heating time, while add forced turning or aeration operations for OC. The similar composition of AMX and AMX-TC resistance bacterial communities indicates that the dominant drug-resistant strains under AMX selection pressure may be easier to obtain or induce the multi-drug resistance. These results provide sufficient data support and better technical assistance for the green application,

widespread promotion and further development of the "toilet revolution", as well as the harmless and environmentally friendly composting and its safe land application.

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