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Changes in antibiotic concentrations and antibiotic resistome during commercial composting of animal manures☆

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ABSTRACT

The over-use of antibiotics in animal husbandry in China and the concomitant enhanced selection of antibiotic resistance genes (ARGs) in animal manures are of serious concern. Thermophilic composting is an effective way of reducing hazards in organic wastes. However, its effectiveness in antibiotic degradation and ARG reduction in commercial operations remains unclear. In the present study, we determined the concentrations of 15 common veterinary antibiotics and the abundances of 213 ARGs and 10 marker genes for mobile genetic elements (MGEs) in commercial composts made from cattle, poultry and swine manures in Eastern China. High concentrations of fluoroquinolones were found in the poultry and swine composts, suggesting insufficient removal of these antibiotics by commercial thermophilic composting. Total ARGs in the cattle and poultry manures were as high as 1.9 and 5.5 copies per bacterial cell, respectively. After thermophilic composting, the ARG abundance in the mature compost decreased to 9.6% and 31.7% of that in the cattle and poultry manure, respectively. However, some ARGs (e.g. *aadA*, *aadA2*, *qacEΔ1*, *tetL*) and MGE marker genes (e.g. *cintI-1*, *intI-1* and *tnpA-04*) were persistent with high abundance in the composts. The antibiotics that were detected at high levels in the composts (e.g. norfloxacin and ofloxacin) might have posed a selection pressure on ARGs. MGE marker genes were found to correlate closely with ARGs at the levels of individual gene, resistance class and total abundance, suggesting that MGEs and ARGs are closely associated in their persistence in the composts under antibiotic selection. Our research shows potential disseminations of antibiotics and ARGs via compost utilization.

1. Introduction

Antibiotics are commonly used in animal husbandry to treat and prevent diseases, as growth promoters and to improve feed efficiency (Jechalke et al., 2014; Sarmah et al., 2006). Generally, the antibiotics fed to the animals are poorly absorbed and large proportions can be excreted into feces as the parent compounds or in bioactive forms (Jechalke et al., 2014; Sarmah et al., 2006). In China, nearly half of the antibiotics consumed (162,000 tons in 2013) were used in animal husbandry, of which substantial amounts could end up in manure (Zhang et al., 2015). Concentrations of some veterinary antibiotics in manures were found to reach hundreds or even thousands of mg kg^{-1} in some parts of China (Zhao et al., 2010). Therefore, direct land applications of these antibiotic-contaminated manures may introduce substantial amounts of antibiotic residues into arable lands.

Although antibiotics are used mainly to control bacterial infections, some bacterial groups play important roles in antibiotic production or in their degradation (Dantas et al., 2008; Martinez, 2008; Sarmah et al., 2006). During these processes, the antibiotic resistant bacteria (ARB) rely on antibiotic resistance genes (ARGs) for protection from the compounds. The widespread use of antibiotics accelerates the development of antibiotic resistance and

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facilitates the maintenance of the resistance at high levels. ARGs could disseminate into a broader range of microbial communities by horizontal gene transfer (HGT) via mobile genetic elements (MGEs), such as plasmids, integrons and transposons (Blair et al., 2014; WHO, 2014). Due to the poorly regulated administration of veterinary antibiotics, China's animal production industries contribute greatly to the enhanced antibiotic resistance in the environmental matrices, such as watercourses and soils (Larson, 2015; Zhu et al., 2013). Assessment of environmental antibiotic resistance relies greatly on the detection of ARGs and gene markers associated with MGEs, such as genes for transposases and class 1 integron-integrase (Gillings et al., 2014; Luby et al., 2016; Zhu et al., 2013).

Manure composting is a common and effective pathway for hazard reduction prior to land application (Bernal et al., 2009). The process has been found to be effective for the degradation of spiked antibiotics in laboratory-scale and pilot-scale studies (Ho et al., 2013; Mitchell et al., 2015; Selvam et al., 2012; Wang et al., 2015). However, little information is available with respect to the levels of antibiotic residues in commercial composts of animal manures in China. Furthermore, the dynamics of ARGs during composting appear to be complex, with a number of studies showing contradictory observations (Selvam et al., 2012; Wang et al., 2015; Zhu et al., 2013). Therefore, investigations on the commercial composts with respect to the antibiotic contents and ARG abundances are necessary, especially in regions with high antibiotic usage, such as Eastern China (Zhang et al., 2015).

In the present study, we determined the concentrations of 15 common veterinary antibiotics using liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) and the abundances of 213 ARGs and 10 MGE marker genes by high-throughput quantitative PCR (HT-qPCR) in the commercial composts made from cattle, poultry and swine manures in Eastern China, during different phases of composting. Correlations between individual ARGs and MGE marker genes and their relationships with each antibiotic were analyzed to reveal possible mechanisms of ARG dissemination.

2. Materials and methods

2.1. Sampling

Manures, thermophilic composts and mature composts were sampled from four large-scale commercial composting companies in Jiangsu province, China, in 2014. Three of the four companies produce aerobic and thermophilic commercial composts exclusively from manures of cattle, poultry and swine, respectively, with the additions of rice straw as the bulking agent. The fourth company uses a mixture of mainly poultry and some other animal manures. All composting operation lasted for about 1 month with a thermophilic phase (temperature > 60 °C) of approximately 2 weeks. Different types of samples were taken on the same day in each company. Manures to be composted were taken from the raw material pools. Composts were sampled at the thermophilic and mature phases during composting according to the method described by Stevens (2010). At each sampling time point, 12 samples were taken from the top, middle, and bottom of the composting piles, mixed thoroughly and subsampled into 3 replicates. All samples from the aforementioned 3 companies contained 3 replicates. These samples were named in the format of animal type (C, P and S for cattle, poultry and swine, respectively), followed by the composting phase (M, T and C for the raw manure, thermophilic compost and mature compost, respectively). Samples from the fourth company that uses mixed manures were taken with just one replicate and named as PM1, PT1 and PC1, and the data were included only for correlation analysis. All samples were kept in ice boxes and transported to the laboratory as soon as possible. Samples were then freeze-dried, homogenized by sieving through a 0.15-mm mesh and stored at −80 °C for further analysis.

2.2. Determination of antibiotics

The concentrations of fifteen antibiotics, including 4 tetracyclines [tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC), doxycycline (DOC)], 4 fluoroquinolones [norfloxacin (NFC), ofloxacin (OFC), ciprofloxacin (CFC), enrofloxacin (EFC)], and 7 sulfonamides [sulfadiazine (SDZ), sulfamethoxazole (SMX), sulfadimidine (SM2), sulfamonomethoxine (SMM), sulfaquinoxaline (SQX), sulfamethoxydiazine (SM), sulfaclozine (SCZ)], were determined using liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) as described previously (Huang et al., 2013). Briefly, 0.2 g freeze dried samples were weighed into 50 mL centrifuge tubes, immersed in 20 mL EDTA-SPB (Sodium Phosphate Buffer), and left to stand overnight. The tubes were shaken in the dark at 200 rpm for 30 min, ultra-sonicated for 15 min, and centrifuged at 3500 g for 10 min. The supernatants were kept in brown glass bottles and the residues were extracted with 10 mL extraction buffer twice more. Supernatants from the 3 extractions were combined, diluted to 500 mL with Milli-Q water (18.2 MΩ cm, Millipore, Bedford, MA), and passed through solid phase extraction cartridges (Oasis HLB, 6 cc/500 mg, Waters, Watford, UK) which had been preconditioned with 10 mL of methanol and 10 mL of super pure water. The cartridges were rinsed with 10 mL super pure water, dried for 20 min with a gentle flow of nitrogen gas, and eluted with 10 mL methanol (supplemented with 0.1% formic acid). The eluents were concentrated with a flow of nitrogen gas, dissolved in 1.0 mL methanol and water (v:v = 3:2, supplemented with 0.1% formic acid), and passed through 0.22 μm membrane filters (Millex, Millipore Corp., Billerica, MA). The solutions were kept in 1.5 mL amber vials before analysis. Antibiotics in the samples were quantified by LC-ESI-MS/MS with calibration standards and internal standards as described by Huang et al. (2013). Internal standards (tetracycline-D5 for tetracyclines, ciprofloxacin-D8 for fluoroquinolones and sufadimethoxine-D6 for sulfonamides) were spiked into the sample matrices (100 μg kg^{-1}) prior to the extraction and into calibration standards (100 μg L^{-1}) (Huang et al., 2013). Average recoveries for tetracyclines, fluoroquinolones and sulfonamides in manures and composts were 96.6 ± 9.3%, 121 ± 18.9% and 95.4 ± 5.0%, respectively.

2.3. DNA extraction and purification

DNA was extracted from 0.3 g freeze-dried and ground samples each time using a PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's instruction. Three to ten aliquots of each sample were extracted and pooled to obtain enough DNA for analysis. The DNA extracts were purified with a PowerClean DNA Clear-up Kit (MoBio Laboratories, Carlsbad, CA). Purification of DNA from swine manure failed to yield enough purified DNA probably due to a high level of impurities in the DNA extracts and relatively low capacity of the PowerClean DNA Clear-up Kit in the cleaning process under this circumstance. For this reason, ARGs could not be determined in the swine manure (SM) samples. The absorbance at 230, 260 and 280 nm of the purified DNA samples was determined by a NanoDrop 2000C spectrophotometer (Thermo Scientific, Wilmington, USA). The quality of the purified DNA was deemed acceptable if A_{260}/A_{280} was greater than 1.8 and A_{260}/A_{230} greater than 1.7. DNA concentrations were measured with a Quant-iT PicoGreen double-stranded DNA