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Long-term manure application increased the levels of antibiotics and antibiotic resistance genes in a greenhouse soil

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ABSTRACT

Land application of animal manure, as an important agronomic practice, represents a potential route to disseminate antibiotic resistance genes (ARGs) into the soil environment. Previous studies have demonstrated that manure-derived ARGs and pathogenic organisms rapidly decline over time following manure application, however, the impacts of long-term and repeated application of animal manure on the diversity and abundance of soil ARGs remain less understood. Here, we investigated the diversity and abundance of ARGs in greenhouse soils with long-term dairy cattle and chicken manure (DM and CM) application, and the half-life of antibiotic resistant bacteria (ARB) isolated from manured soils in Taiyuan, China. Both DM and CM significantly improved the levels of residual antibiotics, the abundances of ARGs, *int*I1 and soil organic carbon and total nitrogen that there was strong link between them. The half-life of ARB was different, and the correlations of ARGs, *int*I1 and *tnp*A genetic elements extracted from ARB with culture times were different. The significantly positive link was between the population of resistance bacteria and the half-life. Our finding suggested that both the levels of antibiotics and the stability of ARGs will determine the diversity and abundance of ARGs in manured soils.

1. Introduction

The synthetic antibiotics are widely used in livestock husbandry for the purposes of infection treatment and growth promotion, which account for more than 70% of the global antibiotic consumption (Jechalke et al., 2014). Since many antibiotics are poorly absorbed and digested in animal bodies, approximately 75% of them are excreted without any degradation through urine and feces (Chee-Sanford et al., 2009), resulting in high concentrations of residual antibiotics commonly detected in animal manures (Fang et al., 2014; Qiao et al., 2012; Zhao et al., 2010). These antibiotic residues can exert strong selection pressure on soil resident microbiome; and at the same time antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) originated from animal manures can also enhance the level of antibiotic resistance in soils, when manures are used as organic fertilizers in agro-ecosystems (Heuer et al., 2011a; Zhu et al., 2013). Therefore, manured soils can be recognized as a rich reservoir of ARGs, which are in potential risk of transmission to other soil bacteria and pathogens through the pathway of horizontal gene transfer mediated by mobile genetic elements (MGEs) (Jechalke et al., 2014). These environmental ARGs can also migrate into the food chain and pose a threat to human health (Marti et al., 2013; Chen et al., 2017). The emerging prevalence of ARGs in environmental settings (Knapp et al., 2010) has caused global concerns, which necessitate an improved understanding of the behaviors of environmental ARGs before development of effective strategies to reduce their dissemination.

Elevated levels of ARGs have been observed across diverse agricultural soil types treated with swine, chicken or dairy manures in various microcosm and field studies (Marti et al., 2013; Schmitt et al., 2006; Udikovic-Kolic et al., 2014; Zhang et al., 2017). The selection pressure imposed by antibiotic residues was considered as the major reason for the observed increasing ARGs in most of these studies. For example, the abundance of tetracycline resistance genes (*tet*) was strongly correlated with the concentrations of tetracycline residues in soils adjacent to swine feedlots (Wu et al., 2010). Long-term manure amendments over decades greatly increased the antibiotic concentrations and ARGs abundances in paddy soils, and the abundance of ARGs was statistically correlated with antibiotics (Tang et al., 2015). The abundance of sulfonamide resistance genes (*sul*) was significantly increased due to application of manure containing sulfadiazine (Heuer

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et al., 2011b). Ross and Topp (2015) reported that land application of dairy manure increased ARG abundance in the bacterial fraction, but not in the phage fraction. The presence of ARGs, however, was sometimes reported to be relatively independent of their corresponding antibiotics, as exemplified by the significant correlations between the abundance of *sul* genes with levels of Cu, Zn and Hg in manures and agricultural soils adjacent to feedlots (Ji et al., 2012), which was attributed to the indirect selection of heavy metal stress other than antibiotics (Hu et al., 2016). Udikovic-Kolic et al. (2014) reported that manure amendment induced a bloom of certain soil ARB that was independent of antibiotic exposure of cows from which the manure was derived. Schmitt et al. (2006) failed to detect a clear influence of manuring on the diversity of *tet* resistance genes in field soils, which might be due to similarity of the resistance gene profiles between manure and soils. Therefore, our understanding of the effect of long-term manure application on the profiles of ARGs and ARB remains elusive.

Apart from the sources and rates of animal manures applied to the field, the effects of manure application on the profiles of ARGs were also dependent on environmental factors. The contribution of manure to the levels of soil ARGs was reported to be dependent on soil background ARGs and soil characteristics (Munir and Xagoraraki, 2011). A lower abundance of ARGs was observed in the manured soils with plants compared with those without plants (Wang et al., 2015). The relative abundances of ARGs increased with the extension of greenhouse planting years, and higher levels of ARGs were found in the manured greenhouse soils than in the field soils (Fang et al., 2014). Manure and sulfadiazine synergistically increased bacterial antibiotic resistance, and the effect of manure was more pronounced in a silt loam soil with respect to *sul*1 gene abundance and frequency of resistance plasmid capture than in a loamy sand soil (Heuer and Smalla, 2007). Therefore, it is imperative to explore the impacts of environmental conditions on the fate of ARGs following land application of animal manure.

When manure-derived ARBs are introduced into soils, it is interesting to explore their temporal patterns in the soil environment without the selection pressure imposed by antibiotics. The capacity of resistance to antibiotics was considered as a “metabolic burden” for ARB, in particular if these ARGs are located in plasmids, and therefore plasmid-bearing ARB grow more slowly than common wild type bacteria, which may lead to extinction of plasmid-bearing ARBs in continuous culture without antibiotics (Stemphens and Lyberatos, 1988). Plasmid structural, segregational and isoform stability is determined by many factors such as plasmid load, copy number, replication patterns, substrate type, medium composition, host background, culture conditions and culture temperature (Summers, 1991; Silva et al., 2009, 2012). However, our understanding of the stability of ARGs in antibiotic resistant isolates without antibiotic pressure remains very limited.

In China, it is estimated that about 97,000 tons of antibiotics was utilized in the livestock industry in 2005 (Li et al., 2013), and 1900 million tons of livestock manure are produced annually (Qiu et al., 2013). Residual antibiotics, ARGs and ARB have been widely detected in manures, soils and crop plants (Wu et al., 2010; Ji et al., 2012; Tang et al., 2015; Wang et al., 2015). Compared with most types of agricultural soils, greenhouse soils usually receive more than twice the amount of animal manures as organic fertilizers. Therefore, we hypothesized that the long-term and repeated application of animal manure will result in enhanced levels of antibiotic residues, ARGs and ARB in greenhouse soils. In this study, we sampled greenhouse soils fertilized with dairy cattle or chicken manures continuously for 13 years, and examined the diversity and abundance of ARGs in collected soil samples, and the stability of ARGs in antibiotic resistant isolates without antibiotic pressure. The objectives of this study were (1) to detect the effects of long-term manure fertilization on the diversity and abundance of ARGs and the levels of residual antibiotics; (2) to determine the stability of ARGs from ARB without antibiotics pressure.

2. Materials and methods

2.1. Soil sampling

Soil samples used in this study were collected from an agricultural greenhouse field (37°41.5′N; 112°32.2′E) with a history of 13-year application of dairy cattle manure (DM) and chicken manure (CM) in Taiyuan, Shanxi province, China. There are three independent plots for each manure-amended treatment. The control soil samples were collected from an abandoned field without known history of manure application adjacent to the agricultural field. Soil is classified into Cinnamon soil. The upper 10 cm of soil was collected from six random locations within each plot and composited into a single bulk sample in September 2016. All the soil samples were sieved to 2 mm, thoroughly homogenized, and divided into two portions. One portion was stored at −80 °C for molecular analysis, and the other portion was air-dried for chemical analysis. Soil pH in dH_2O was measured at a soil:solution ratio of 1:5 (w:v). Soil organic carbon (SOC) was determined using the dichromate oxidation method, and total nitrogen (TN) was analyzed by the Kjeldahl method. Soil inorganic nitrogen (nitrate and ammonium) was extracted by 2 M KCl solution and determined by the Random-Access Analyzer (DeChem-Tech GmbH, Germany).

2.2. Extraction and determination of antibiotics

The antibiotic residues were extracted from the manured soil samples using the procedures as described previously (Fang et al., 2014). The concentrations of extracted antibiotics were measured following the method as described in Wang et al. (2015). A total of seven antibiotics including oxytetracycline (OTC), chlortetracycline (CTC), cefpiramide (CPM), cefuroxime (CUM), sulfamethoxazole(SMX), norfloxacin (NOR), and erythromycin (ETM) were analyzed in this study. We selected these antibiotics for detection as they represent the commonly used antibiotics in chicken and cattle feedlots.

2.3. Quantitative PCR (qPCR) analysis of ARGs

Total genomic DNA was extracted from 0.2 g of fresh soils using the MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's protocol. The concentration and quality of the extracted DNA were determined using Infinite 200 PRO (TECAN, Sweden). The qPCR analysis was performed on the iCycler iQ5 thermocycler (Bio-Rad, Herculers, CA, USA) to quantify 28 ARGs including 15 tetracycline resistance genes (*tet*A, *tet*C, *tet*E, *tet*K, *tet*L, *tet*A/

Table 1
Soil pH, organic carbon (SOC), total, available and ammonium nitrogen (TN, AVN and AMN) from unfertilized soil (CK), and dairy cattle- and chicken- manured soils (DM and CM).

	Soil pH	SOC (g/kg)	TN (g/kg)	AVN (mg/kg)	AMN (mg/kg)
CK	7.89 ± 0.06a	15.95 ± 1.50a	0.94 ± 0.08a	11.57 ± 0.58a	85.02 ± 7.25b
DM	7.85 ± 0.04a	23.35 ± 1.90b	1.76 ± 0.10b	12.09 ± 0.42a	66.05 ± 3.80a
CM	7.83 ± 0.12a	21.15 ± 1.81b	2.09 ± 0.33b	19.15 ± 1.50b	74.71 ± 1.01ab

Data are means standard ± deviations. The different case letters indicate that the means are significantly different among soils ($P < 0.05$) with Duncan test.