Temporal changes of antibiotic-resistance genes and bacterial communities in two contrasting soils treated with cattle manure

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ABSTRACT

The emerging environmental spread of antibiotic-resistance genes (ARGs) and their subsequent acquisition by clinically relevant microorganisms is a major threat to public health. Animal manure has been recognized as an important reservoir of ARGs; however, the dissemination of manure-derived ARGs and the impacts of manure application on the soil resistome remain obscure. Here, we conducted a microcosm study to assess the temporal succession of total bacteria and a broad spectrum of ARGs in two contrasting soils following manure application from cattle that had not been treated with antibiotics. High-capacity quantitative PCR detected 52 unique ARGs across all the samples, with β-lactamase as the most dominant ARG type. Several genes of soil indigenous bacteria conferring resistance to β-lactam, which could not be detected in manure, were found to be highly enriched in manure-treated soils, and the level of enrichment was maintained over the entire course of 140 days. The enriched β-lactam resistance genes had significantly positive relationships with the relative abundance of the integrase intI1 gene, suggesting an increasing mobility potential in manure-treated soils. The changes in ARG patterns were accompanied by a significant effect of cattle manure on the total bacterial community compositions. Our study indicates that even in the absence of selective pressure imposed by agricultural use of antibiotics, manure application could still strongly impact the abundance, diversity and mobility potential of a broad spectrum of soil ARGs. Our findings are important for reliable prediction of ARG behaviors in soil environment and development of appropriate strategies to minimize their dissemination.

Keywords: Antibiotic resistance gene; cattle manure; class 1 integron; β-lactamase; soil resistome

INTRODUCTION

The emerging prevalence of antibiotic-resistance genes (ARGs) in clinical and environmental settings has been recognized as one of the most serious threats to the health and welfare of humans and animals in the 21st century (Udikovic-Kolic et al. 2014; Berendonk et al. 2015). The abundance of ARGs in archived environmental samples showed an exponentially increasing trend over time since 1940 (Knapp et al. 2010), owing to the industrial
production of antibiotics and pervasive antibiotic use in hospitals and livestock farms (Allen et al. 2010). The recently released publication from the World Health Organization (WHO) warned that antibiotic-resistant bacteria are now prevailing in many parts of the world (WHO 2014), enhancing the potential risks of causing meningitis and infections of the skin, blood, kidneys and other organs to humans and animals. Therefore, ARGs have been recognized as an important environmental contaminant of global concern (Pruden et al. 2006). Along with the rising burdens of ARGs, it was reported that environmental ARGs could directly transfer from environments to humans, through their persisting and spreading in the environment and subsequent dissemination into the food chain (Forsberg et al. 2012; Zhu et al. 2013). Furthermore, the transfer of ARGs from environmental bacteria to human-associated pathogens could be facilitated through mobility elements such as integrons, plasmids and transposons (Gogarten and Townsend 2005; Binh et al. 2008). Despite the clinical importance of antibiotic resistance (Levy 1997), however, environmental reservoirs of ARGs and their potential contribution to clinical resistance remains less understood (Allen et al. 2009). Therefore, it is essential to identify the sources and behaviors of ARGs in the environment enabling the design of appropriate strategies to reduce the dissemination of ARGs.

To date, the environmental occurrence of antibiotics and ARGs in wastewater, reclaimed water, sludge and sediments has been documented (Auerbach, Seyfried and McMahon 2007; Ghosh and LaPara 2007; D’Costa et al. 2011; Leung et al. 2012; Liu et al. 2012); however, evidence remains largely elusive regarding the spatial and temporal patterns of soil ARGs under anthropogenic disturbance (D’Costa et al. 2006; Forsberg et al. 2014). Soil is considered to be the largest environmental reservoir comprising as much as 30% of the known ARGs in public repositories (Nesme et al. 2014), and to be one of the most complex ecosystems in terms of microbial diversity and ecological niches (Nesme and Simonet 2014). As the only ecosystem interacting constantly with all compartments of the biosphere, soil is prone to genetic exchange by means of horizontal gene transfer between ecologically distinct lineages found in other ecosystems (Nesme and Simonet 2014). High-throughput functional metagenomic analysis found that soil bacteria harbor resistance gene cassettes against all major classes of antibiotics with high similarity to genes from human pathogens (Forsberg et al. 2012). The spread and aggregation of ARGs in soil environments have been strongly correlated with intensive antibiotic use by an extensive body of studies (Binh et al. 2008; Heuer, Schmitt and Smalla 2011a), and might have been accelerated by anthropogenic activities such as agricultural practices, animal husbandry and manure application (Allen et al. 2010). Therefore, assessment of the persistence of ARGs in soil ecosystems and the conditions under which they could be horizontally transferred is critical to prevent future dissemination of soil ARGs into the clinical environments.

Antibiotics are widely used in agriculture for treatments of sick animals, infectious disease prophylaxis and growth promotion, accounting for at least half of the antibiotics produced worldwide (Ghosh and LaPara 2007; Allen et al. 2010; Hu, Zhou and Luo 2010). However, most of antibiotics are poorly absorbed by animals and a large proportion (30%-90%) of them can be excreted and dispersed into soils when manure is applied as fertilizer (Zhang and Zhang 2011). Land application of manure from antibiotic-treated animals was reported to frequently increase the levels of antibiotics and ARGs in soils (Ghosh and LaPara 2007; Martinez 2008; Storteboom et al. 2010; Jechalke et al. 2014b), and these ARGs are subject to further dispersal via leaching to waters, runoff into rivers and sediments, air dust and delivery of agricultural products (Pruden, Arabi and Sorteboom 2012; Zhu et al. 2013). Most of these studies attributed the increasing prevalence of ARGs in manure-treated soils to direct selection of soil ARGs exerted by bioavailable antibiotics in manure (Ghosh and LaPara 2007; Heuer et al. 2011b). However, it was found that the extractable concentrations of antibiotics like sulfadiazine in manure rapidly declined or dissipated after application to soils within 30 days (Hammesfahr et al. 2008; Kopmann et al. 2013). Therefore, the selective pressure of residual antibiotics will rapidly vanish over time, and other mechanisms might exist to influence the soil resistance patterns in the absence of antibiotic selection pressure. In fact, previous studies have suggested that manure amendment of soil adds a considerable amount of manure-derived ARGs on transferable plasmids (Heuer, Schmitt and Smalla 2011a), even if the producing animals have never been treated with antibiotics (D’Costa et al. 2011; Udikovic-Kolic et al. 2014). Resident soil antibiotic-resistant bacteria were also found to significantly increase following application of manure from cows without antibiotic treatment history (Udikovic-Kolic et al. 2014). Therefore, compared with the intensively investigated effects of manure from antibiotic-treated animals (Jechalke et al. 2014a), the empirical evidence is scare regarding the fate of ARGs derived from antibiotic-free manure, potential co-selection of antibiotic resistance and the impact of manure application on soil resistome.

The main objective of this study was to explore how the composition and resistance profiles of soil bacterial community will change over time in microcosm incubations of two contrasting soils following manure application from cattle receiving no antibiotic treatment. High-throughput quantitative PCR (qPCR) arrays were employed to target a broad spectrum of ARGs conferring resistance against the major classes of antibiotics. The class 1 integrase gene (intI1) and a transposase gene (tnpA) that have been described to have an important role in dissemination of resistance (Zhu et al. 2013; Gillings et al. 2015) were quantified as a proxy for mobility potential of ARGs. We hypothesized that: (i) the populations carrying these ARGs might respond differently to manure amendment during the course of incubation, owing to the differential responses of microbial populations to nutrient addition; (ii) manure application would enhance the horizontal gene transfer potential of ARGs, as measured by the abundances of the intI1 and tnpA genes and (iii) changes in ARGs profiles might be paralleled by changes of the total bacterial community, which has been recognized as a primary determinant of soil ARG content (Forsberg et al. 2014).

**MATERIALS AND METHODS**

**Soil and manure sampling**

Soil samples were taken in November 2014 from two distinct sites including an arable field (a vegetable farm) at Clyde (38°07’S, 145°19’E) and a pasture field at Dookie (36°25’S, 145°19’E), Victoria, Australia. The arable field is planted with celery, and has been treated with chicken manure and inorganic fertilizers in the previous years. According to Australian Chicken Meat Federation, there are strict codes of practice in Australia concerning antibiotic use—they can only be used for disease control (therapeutic purposes) but not as growth promoters. Therefore, although we did not measure the antibiotic residue in the chicken manure applied in the vegetable farm, we believe that the chicken was not routinely administrated with antibiotics. The pasture field is adjacent to the Dookie farm (The University of Melbourne), and has no known history of manure application.
Table 1. Basic properties of the two contrasting soils collected from Victoria, Australia.

<table>
<thead>
<tr>
<th></th>
<th>Arable soil</th>
<th>Pasture soil</th>
</tr>
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<tbody>
<tr>
<td>Location</td>
<td>38°07' S, 145°19'E</td>
<td>36°25' S, 145°19'E</td>
</tr>
<tr>
<td>Texture</td>
<td>Loamy Sand</td>
<td>Fine Sandy Loam</td>
</tr>
<tr>
<td>pH (H2O)</td>
<td>7.19</td>
<td>5.06</td>
</tr>
<tr>
<td>Total carbon (%)</td>
<td>3.75</td>
<td>5.06</td>
</tr>
<tr>
<td>Total nitrogen (%)</td>
<td>0.43</td>
<td>0.53</td>
</tr>
<tr>
<td>NH4+ -N (µg g⁻¹)</td>
<td>8.63</td>
<td>10.2</td>
</tr>
<tr>
<td>NO3⁻ -N (µg g⁻¹)</td>
<td>50.0</td>
<td>28.5</td>
</tr>
</tbody>
</table>

application, but the potential influence of human activities on present ARG profiles cannot be excluded. The average annual rainfall at the Clyde site is 819 mm, with the mean annual temperature at 19.4°C. The average annual rainfall at Dookie is 551 mm, and the mean annual temperature is 20.9°C. The soils are classified as Loamy Sand at Clyde, and as Fine Sandy Loam at Dookie. The detailed information about the soil properties is listed in Table 1. The control soil samples were taken from pristine forests in two remote national parks away from the Melbourne city: Lake Eildon National Park (37°13’S, 144°52’E) and Yarra Ranges National Park (37°41’S, 145°59’E), Victoria, Australia. The two national parks have no known exposure to antibiotics and have minimal human-induced selective pressure. At each site, the top 15 cm of surface soils were collected, thoroughly homogenized and sieved through a 2.0 mm mesh, and transported on ice to the laboratory. The cattle manure used in this study was taken from pens (20 m × 20 m) of cattle at the Dookie campus (The University of Melbourne), which had not been treated with antibiotics or heavy metal compound as feed additives and is well-separated (~2 km) from the dairy farm. The manure samples were collected within 3 days after excretion, and transported on ice to the laboratory within 3 h of sampling.

Soil microcosm incubations

Soil and manure samples taken from the two sites were immediately used for a microcosm incubation. Soil moisture contents were adjusted to 60% of the water-filled pore space. Manure was thoroughly mixed with sieved soils to achieve two levels of fertilizer: 40 and 80 mg g⁻¹ soil, which corresponded to typical agricultural amounts of 30 and 60 m² manure ha⁻¹, respectively. Three sets of treatments were established in triplicate microcosms for both soils: the untreated soil (U), a low level of manure application at 40 mg g⁻¹ soil (LM) and a high level of manure application at 80 mg g⁻¹ soil (HM). Microcosms were established in 500 ml vials with 20 g of soils or manured soils. The loosely covered soil microcosms were maintained constant at 20°C in the dark, and destructively sampled at five time points on days 1, 35, 63, 91 and 140 post manure application. Aerobic conditions in the vials were maintained by opening microcosms for air refreshing and the water loss was replenished weekly throughout the incubation.

Soil physicochemical analysis and DNA extraction

Soil total nitrogen and total carbon were measured using the classic Dumas method of combustion on the isotope-ratio mass spectrometry (Sercon Hydra, Crewe, UK). Soil and manure pH values were measured using a ratio of 2.5 (water to soil/manure) with an Orion Star A211 pH Meter (Thermo Scientific Inc., Melbourne, Australia). Soil ammonium and nitrate were extracted with 2 M KCl and determined by a Continuous Flow Analyzer (SAN++, Skalar, Breda, Holland). The total genomic DNA was extracted from 0.5 g of soil or manure samples by using the MoBio PowerSoil DNA Isolation kit (MoBio Laboratories, Carlsbad, CA, USA) ’as per’ the manufacturer’s instructions, with slight modifications that the initial cell-lysis step was achieved by using a FastPrep bead beating system (Bio-101, Vista, CA, USA) at a speed of 5.5 m s⁻¹ for 30 s. The extracted DNA was further purified using the Wizard DNA Clean-Up System (Promega, San Luis Bispo, CA, USA). The DNA concentration and quality were assessed using the NanoDrop ND2000c Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The A260/A280 ratios were greater than 1.8 for all the manure and soil DNA extracts.

High-throughput profiling of ARGs by qPCR arrays

The occurrence and temporal changes of ARGs were analyzed using the Antibiotic Resistance Genes Microbial DNA qPCR arrays (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions, enabling high PCR specificity and accurate quantification. The current version of array can simultaneously target a broad-spectrum profile of 84 genes from all major classes of ARGs (Table S1, Supporting Information), including aminoglycoside, β-lactam (Four Ambler classes A, B, C and D are treated separately depending on their primary structure (Jacoby and Munoz-Price 2005)), erythromycin, quinolone and fluoroquinolone, macrolide-lincosamide-streptogramin_b (MLSb), tetracycline, vancomycin and multidrug-resistance classifications. The targeted ARGs are associated with the antibiotics intensively used as veterinary and human medicine, or growth promoters.

The 25 µl reaction mixture consisted of 12.5 µl of HotStart DNA Polymerase Mastermix (Qiagen), 5–10 ng of template DNA, 1 µl of bovine serum albumin (20 µM) and microbial DNA-free water. The mixtures were aliquoted into each well of the 96-well array plate containing a mix of two pre-dispensed, gene-specific primers and one fluorescent hydrolysis probe. Pan-bacteria assays were included as positive controls for the presence of bacterial DNA, and the positive PCR control assay is also included to test for PCR inhibitors and the efficiency of qPCR runs using a pre-dispersed artificial DNA sequence and the primers that detect it. Thermal cycling was performed on a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) as follows: an initial PCR activation step at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C for denaturation and 2 min at 60°C for annealing and extension. The baseline and threshold fluorescence values were manually adjusted to the same levels across all qPCR runs, and a threshold cycle (Ct) value of 37 was used as the detection limit. The Ct values generated from the qPCR runs were imported into Data Analysis Template Excel Software (Qiagen) to calculate the fold change values of all ARGs. The ΔΔCt method of relative profiling was conducted to evaluate the temporal dynamics of ARGs in soil samples as described previously (Zhu et al. 2013).

qPCR analysis of the intI1, tnpa and bacterial 16S rRNA genes

Abundance of the intI1 gene (an integrase gene of class 1 integrons), tnpa (a transposase gene of the IS6 family transposons) was quantified on a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad) using the primer sets HS463a/HS464.
(Hardwick et al. 2008) and tnpA-04F/tnpA-04R (Zhu et al. 2013; Wang et al. 2014), respectively. The total bacterial 16S rRNA gene was also quantified using the BACT1369F/PROK1492R with the probe TM1389F (Suzuki, Taylor and DeLong 2000). Amplification was performed in a total volume of 10 μl including 5 μl of SYBR Premix Ex Taq (TaKara Biotechnology, Otsu, Shiga, Japan), 0.5 μl of each primer (10 μM), and 2 μl of 10-fold diluted template DNA. Standard curves for quantification were generated from preparing 10-fold serial dilutions of plasmids containing correct inserts of the target genes. The specificity of PCR amplicons was verified by melting curve analysis following each qPCR run. PCR efficiency ranged between 85% and 96% for all the qPCR runs. The relative abundance of the intI1 and tnpA genes was calculated by normalizing to the bacterial 16S rRNA gene abundance to compensate for variance induced by differential DNA extraction efficiency and amplification efficiency across samples.

Community profiling of the bacterial 16S rRNA gene by terminal restriction fragment length polymorphism

Changes in the total soil bacterial community structure before and after the manure treatment were determined by the terminal restriction fragment length polymorphism (T-RFLP) analysis of the bacterial 16S rRNA genes using the fluorescently labeled primers FAM-27F/1492R (Weisburg et al. 1991). The 50 μl PCR mixture contained 2.5 U of BioTaq DNA polymerase (Bio-line, Sydney, NSW, Australia), 5 μl of 10 × NH₄ reaction buffer, 2 μl of MgCl₂ solution (50 μM), 1 μl of each primer (10 μM), 1 μl dNTP mix (20 μM) and 2 μl of template DNA (1–10 ng). Thermal cycling conditions were as follows: 5 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 56 °C, and 1 min at 72 °C, followed by 72 °C for 10 min. The PCR products were purified using the Wizard SV Gel and PCR Clean-Up System before digestion with restriction endonuclease HhaI (BioLabs, Sydney, NSW, Australia). Digests were incubated at 37 °C for 3 h, followed by 10 min at 95 °C to deactivate the restriction enzyme. Terminal restriction fragments (TRFs) were resolved on an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems, CA, USA), and processed using Genemapper version 4.0 (Applied Biosystems) as described previously (Hu et al. 2015, 2016). The relative fluorescence abundance of all TRFs was exported for downstream analysis.

Network analysis and visualization

The co-occurrence patterns between different types of ARGs detected using the high-throughput qPCR array were explored in network analysis using the CoNet Cytoscape plug-in (Soffer, Zaneveld and Thurber 2014). Briefly, for all the pairwise interactions, correlation scores were calculated using Pearson correlation, Spearman correlation, mutual information, Bray–Curtis dissimilarity and Kulback–Leibler dissimilarity. All ARG types below a minimum occurrence of 3 across all the samples were discarded to avoid introduction of spurious correlations. The Re-Boot procedure with 100 permutations was conducted to control the potential false-positive correlations, and the resultant distribution was further refined with 1000 bootstraps. The P values for correlations were combined from the five correlation measures using the Simes method, and only correlations found to be significant by at least two correlation methods were included (Soffer, Zaneveld and Thurber 2014). The resultant pairwise correlations between the ARG types were utilized to construct the co-occurrence networks. Network topology was visualized and explored using the Frucherman Reingold algorithm on the open-source interactive platform Gephi (Bastian, Heymann and Jacomy 2009). Only correlations with a P-value above 0.8, and a significance level below 0.05 were displayed (Junker and Schreiber 2008).

Statistical analysis

The relative abundance of intI1 and tnpA genes was log-transformed prior to statistical analysis to meet normality assumptions. A two-way analysis of variance (ANOVA) was performed to analyze the effects of manure application and sampling time on the intI1 and tnpA gene abundance. Linear regression analysis was conducted to relate the relative abundance of intI1 and tnpA genes to the fold change of ARGs based on the log-transformed data. Statistically significant differences were accepted at P < 0.05. Non-metric multidimensional scaling (NMDS) analysis was performed to visualize the Euclidean dissimilarity matrix based on the relative abundance of ARGs and to visualize the Bray–Curtis dissimilarity matrix based on the relative abundance of the bacterial 16S rRNA gene TRFs. Additionally, the effects of treatments and sampling time on the patterns of ARGs and bacterial communities were tested by two-way permutation multivariate analysis of variance (PERMANOVA), by using the ‘adonis’ function in the ‘vegan’ package of the R platform with 999 permutations. The heat maps illustrating the qPCR array results of ARGs with log-transformed fold changes were generated using the ‘gplots’ package in R.

RESULTS

Broad-scale screening of diverse ARGs in manure and soil samples

Among all the manure and soil samples, a total of 52 unique ARGs were detected, compared with 16 ARGs found in the control samples from the remote national parks. The untreated arable soil harbored a remarkably higher number of ARGs than those detected in the untreated pasture soil and the control pristine soil, but was comparable to the cattle manure samples (Fig. 1a). Manure application significantly increased the number of ARGs detected in the pasture soil, with a slight (but not significant) effect observed in the arable soil (Fig. 1a). The ARGs detected across the untreated and manured samples in all the sampling points could potentially confer resistance to a broad spectrum of antibiotics (Fig. 1b). The β-lactam resistance genes including classes A, B, C and D were the most frequently detected ARGs, comprising 55.8% (an average of all sampling time points) of the total number of ARGs. Other frequently detected ARGs included resistance genes for quinolones and fluoroquinolones (11.5%), aminoglycoside (9.6%) and MLS β (9.6%). The cattle manure encompassed divergent ARGs profiles from the control, untreated and manure-treated soil samples, with a significantly lower proportion (27.7%) of β-lactam resistance genes found in the manure samples, compared to 36.6% and 42.9% in untreated arable and pasture soils, respectively (Fig. 1c). The compositions of ARGs were quite similar between the two soils, and no significant effects of manure application on soil ARG compositions in terms of the number of each class of ARGs detected in soil samples were observed (Fig. 1c).

Temporal changes in the relative abundance of diverse ARGs following manure treatments

The prevalence and abundance of ARGs in untreated and manure-treated soils were assessed at five time points during
the microcosm incubations using the high-resolution qPCR arrays, which demonstrated positive signals for a wide variety of ARGs (Fig. 2). The untreated arable soil had higher detection frequencies of ARGs than the untreated pasture soil, and the relative abundance of these detected ARGs stayed largely unchanged during the incubation. However, manure application significantly altered the relative abundance of ARGs in both soils (Fig. 2), and the changes in ARG patterns could be generally classified into two different categories: (i) several genes conferring resistance to β-lactam (Per-1, Per-2, IMP-2 and IMP-5 groups in the arable soil, and Per-1, SHV, IMP-2 and IMP-5 groups in the pasture soil), which could not be detected in cattle manure, remarkably increased their relative abundance in both soils at early time points following manure treatment towards day 63. The relative abundance of these genes tended to decrease to the pre-treatment level in the arable soil at day 140 (Fig. 2a), but were maintained at a high level in the pasture soil (Fig. 2b) until the end of the incubation; (ii) ARGs conferring resistance to aminoglycosides, MLS_b and tetracycline were abundant in manure samples but not frequently observed in the pasture soil (Fig. 2b). Manure application in the pasture soil resulted in an obvious increase in the abundances of these ARGs, and the level of enrichment was maintained until the end of the incubation.

NMDS analysis based on the Euclidean dissimilarity matrix revealed a clear divergence of the manure samples from the untreated and manured soil samples for both soils (Fig. 3). The untreated soil samples clustered separately from the samples treated with manure, and the ARGs in the later stage of incubation (days 91 and 140) tended to be closer to the untreated soils samples, suggesting a resilience of the bacterial communities harboring ARGs from manure disturbance. The two-way PerMANOVA test indicated that patterns of the relative abundance of ARGs were significantly different across the three treatments (i.e. untreated, low-level manure and high-level manure) for both soils, and were also significantly influenced by the interaction of treatment × sampling time (Table S2, Supporting Information).
Effects of manure application on the relative abundance of \textit{intI1} and \textit{tnpA} genes

To provide evidence for the mobility potential of ARGs under manure application, the class 1 integrase \textit{intI1} gene and the transposase \textit{tnpA} gene were quantified from both manure and soil samples. The \textit{intI1} gene abundance relative to the bacterial 16S rRNA gene, ranging from $-3.28$ to $-2.54$ log units in the arable soil to $-3.96$ to $-2.02$ log units in the pasture soil, was more than two orders of magnitude higher than the \textit{tnpA} gene abundance ranging from $-5.80$ to $-2.52$ to $-5.92$ to $-3.89$ in the arable and pasture soils, respectively (Fig. 4). Significantly positive effects of manure application and sampling time were found on the relative abundance of \textit{intI1} in the arable soil, with the maximal \textit{intI1} gene copies observed at day 63 (Fig. 4a). Over the course of 140 days’ microcosm incubation, the relative abundance of \textit{intI1} in manure-treated soils decreased reaching values in the untreated soils. A similar temporal pattern of \textit{intI1} could be found in the pasture soil (Fig. 4b), albeit no significant effects of treatment or time could be found. By contrast, the \textit{tnpA} gene abundance tended to slightly fluctuate over time in the arable soil (Fig. 4c), and remained largely stable for all the treatments in the pasture soil (Fig. 4d). No significant effects of treatment or time could be observed on the \textit{tnpA} gene abundance in the two soils.

Spearman’s correlation analysis was further performed to explore the potential for horizontal gene transfer of ARGs by relating all types of ARGs to the relative abundance of the \textit{intI1} gene.
indicating that these genes might be concomitantly harbored in some specific microbial groups or some specific mobile genetic elements (even in various microbial groups).

**Effects of manure application on total soil bacterial communities**

The changes in ARG patterns suggested that soil bacteria carrying these ARGs might be also changed simultaneously, which prompted us to explore the responses of soil bacterial community structure to manure application. The T-RFLP analysis of the bacterial 16S rRNA gene by the HhaI enzyme yielded 28 distinct TRFs across all the treatments, of which TRF-191, TRF219, TRF-235, TRF-337 and TRF-365 were the most prominent phylotypes. The temporal patterns of bacterial communities were similar to those of ARGs, with the cattle manure clearly separated from the soil and manured soil samples for both soils (Fig. 7). The bacterial community composition in soils treated with cattle manure differed significantly from the untreated soils, which was corroborated by two-way PerMANOVA analysis ($P < 0.05$) for both soils (Table S2, Supporting Information). The effects of sampling time and the interaction of treatment $\times$ sampling time on the soil bacterial communities were also found to be significant in the arable soil (Table S2, Supporting Information).

**DISCUSSION**

**Diversity and abundance of ARGs in soil and manure samples**

Soil is the original habitat of most currently known antibiotics (Wright 2010), thus soil bacteria could have developed resistance even if various antibiotics had not been produced since the 1940s (Davelos, Kinkel and Samac 2004). Genomic studies suggested that soil microorganisms (e.g., the phylum Actinobacteria) have the potential to produce a wide variety of bioactive compounds, some of which can be identified as antibiotics (Allen et al. 2010). Therefore, it is not surprising to detect a set of diverse ARGs in both untreated soils and the control pristine soils (Figs 1 and 2), and these ARGs represent nearly all classical resistance mechanisms including antibiotic efflux, target protection, and antibiotic inactivation (Walsh 2000). In line with previous screening of soil ARGs (Li et al. 2015), genes conferring resistance to aminoglycoside, $\beta$-lactam, quinolones and fluoroquinolones and MLS$_b$ were found to be highly abundant in our soil samples. The average number of detected ARGs in the arable soil with known history of chicken manure amendment was significantly higher than that in the pasture soil and the control pristine soils, possibly reflecting the strong impact of anthropogenic activities in altering soil reservoirs of ARGs. Although Australia has strict codes of practice concerning antibiotic use in chicken farm, we still cannot exclude the possibility of the therapeutic use of antibiotic for disease control and its subsequent selective pressures on ARGs in chicken manure and the arable soils.

Animal manure is widely used to improve soil fertility levels but is also recognized as a substantial carrier of ARGs and pathogens into soil (Zhu et al. 2013; Wichmann et al. 2014). Amendment of soils with manure from antibiotic-treated animals has been frequently reported as an important route by which ARGs enter the environment and food system (Ghosh et al. 2007; Heuer et al. 2008). However, recent studies suggested that high frequencies of ARGs (such as $\beta$-lactamases and tetracycline-resistance genes) are encountered in manure from dairy cows receiving no administration of veterinary antibiotics.
Figure 4. Time-course changes of the relative abundance of the intI1 gene targeting class 1 integrons for the arable soil (a) and pasture soil (b). The graph also shows time-course changes of the relative abundance of the trpA gene targeting transposase for the arable soil (c) and the pasture soil (d). Error bars indicate standard errors \((n = 3)\). (Abbreviations: U, untreated soil samples; LM, low-level manure treatment; HM, high-level manure treatment; M, manure samples.)

(Kyselkova et al. 2013; Udikovic-Kolic et al. 2014). Similarly, the cattle manure samples used in this study, which have not been treated with antibiotics, were also found to be a major source of diverse and abundant ARGs. The average number of ARGs detected in manure samples was comparable to that in the arable soil with history of chicken manure fertilization, but the composition was slightly different with a smaller proportion of \(\beta\)-lactam resistance genes found in the cattle manure (Fig. 1c). The resistance genes for aminoglycosides, MLS\(_B\) and tetracycline (tetA and tetB) were highly abundant in manure samples (Fig. 2), suggesting that bacteria resistant to these antibiotics are present in gastrointestinal tracts of cattle that received no administration of antibiotics. These antibiotic-free manure samples have divergent ARG profiles from the swine and cow manure receiving large amount of antibiotics, in which \(\beta\)-lactam, kanamycin and macrolide resistance genes were primarily enriched (Zhu et al. 2013; Wichmann et al. 2014). Although manure samples can be strikingly different between our study and others’ regardless of the history of antibiotic treatment and husbandry practice, we provide further evidence that even in the absence of selective pressure imposed by agricultural use of antibiotics, clinically relevant ARGs are ubiquitous and abundant in cattle manure samples. Therefore, studies on the co-selection of heavy metal resistance and antibiotic resistance, and impacts of disinfectants like quaternary ammonium compounds on manure ARGs will be highly desirable in future studies. The differences in ARGs profiles between manure and soil samples necessitate the detailed assessment of the fate of manure-derived ARGs and the impacts of manure application on soil indigenous ARGs.

Temporal patterns of soil ARGs and stimulation of \(\beta\)-lactam-resistance genes following manure application

The abundant and diverse ARGs found in antibiotic-free cattle manure are in line with the similar findings in previous studies (Looft et al. 2012; Kyselkova et al. 2013, 2015; Wichmann et al. 2014), which prompted us to explore the evolution and movement of these ARGs after manure application. Although land application of cattle manure is a common practice in crop production, current knowledge regarding the temporal patterns of ARGs following manure amendment is limited, which hinders our ability to reliably predict ARG behaviors in soil environment. In this microcosm study, by incubating two contrasting soils amended with two levels of cattle manure, we observed different temporal patterns of ARGs over the course of 140 day’s incubation. A key finding from this study is that the relative abundance of several \(\beta\)-lactamas (native in untreated arable soil but below the detection limit in cattle manure) significantly increased after manure treatment and then gradually decreased to the pre-treatment levels (Fig. 2a). By contrast, the relative abundance of these \(\beta\)-lactamases (native
Our data are supported by similar results in a previous study which found that the CEP-04 group belonging to β-lactamases was selected in an agricultural soil fertilized with antibiotic-free cow manure, and β-lactamases found in soil bacteria were not amplified from dairy cow manure (Udikovic-Kolic et al. 2014). Functional metagenomic analysis attributed the enrichment of β-lactamases in manure-treated soil to the growth of soil resident genus Pseudomonas sp. harboring β-lactamases, which is commonly found in soil environment (Udikovic-Kolic et al. 2014) and particularly responsive to manure amendment (Ding et al. 2014). These findings indicated that amendment of soil with antibiotic-free manure will more likely lead to the proliferation of bacteria carrying β-lactamases possibly originating from the soil samples rather than introduced from manure-derived ARGs, although different β-lactamases were enriched in different soils indicating a soil type specific effect. Moreover, manure-treated soil was found to harbor a higher abundance of β-lactamases than soil treated with inorganic fertilizer (Udikovic-Kolic et al. 2014), suggesting that some particular forms of nutrients contained in manure may provide a competitive advantage for the growth of soil resident bacteria carrying β-lactamases. Aside from nutrients in manure, many other factors such as heavy metals that are widely used as feed additives for animals, could also contribute to the emergence, co-selection and dissemination of diverse environmental ARGs (Kyselkova et al. 2015), including β-lactamases (Nesme and Simonet 2014), but this is not a case in our study, because no metal compounds were added to the feed for cattle from which the manure samples were collected.

Beyond the potential impact of manure on soil resident microbial communities, other mechanisms could be also existing in this study, such as introduction of manure-derived ARGs and microorganisms (Heuer et al. 2011b). Manure and soil were reported to harbor distinct bacterial communities (Hammesfahr et al. 2008; Heuer et al. 2008), therefore manure has a pronounced effect on soil bacterial community structures by introducing manure-derived microbes, as evidenced by the T-RFLP fingerprinting (Fig. 7). We found that when ARGs conferring resistance to aminoglycosides, MLSβ and tetracycline were highly abundant in manure samples but not in the pasture soil (Fig. 2b), manure application could introduce a pulse of these ARGs. By contrast, when ARGs conferring resistance to aminoglycosides, MLSβ and tetracycline were abundant in both manure samples and the arable soil (Fig. 2a), manure amendment did not obviously change the patterns of these genes. Therefore, we supposed that although the manure-derived ARGs could be temporally enriched in soils, particularly in the pasture soil without history of manure application, they cannot become a major component in soil environment over time. Previous studies also suggested that manure-derived bacteria could not thrive in soil environment, and gradually decreased after manure treatment (Hammesfahr et al. 2008; Heuer et al. 2008), which was attributed to the competition with resident soil bacteria and the differences in environmental conditions between soil and animal gut (Chee-Sanford et al. 2009). Most of the gut microbiota from animals and humans are restricted to growth under anaerobic conditions (Goodman, Kallstrom and Faith 2011), which are highly different from the aerobic conditions in our microcosms. Therefore, it might be difficult for microorganisms in the animal gut to be adapted to living in the soil environment. The manure-derived bacteria have to transfer their ARGs to soil indigenous bacteria or pathogens for the prevalence of these ARGs in soil environments.

Figure 5. Correlations between the fold changes of β-lactam-resistance genes and the relative abundance of the intI1 gene in the arable soil (a) and pasture soil (b). All the data were log-transformed before the Spearman’s correlation analysis. None of the β-lactam resistance genes displayed here were detected in the cattle manure samples.
Potential for horizontal gene transfer of ARGs in manure-treated soils

The high abundance and diversity of ARGs observed in the manure-treated soils (Fig. 2) provide a high probability of dispersal and mobility in soil environments and a subsequent possibility of these ARGs being transferred to human pathogens. The horizontal gene transfer from manure to indigenous soil bacteria might be important for resistance dissemination to persist in soil (Chee-Sanford et al. 2009; Gillings and Strokes 2012), because bacteria from manure are not sufficiently adapted to soil environments (Chee-Sanford et al. 2009; Heuer, Schmitt and Smalla 2011a). Most of ARG cassettes are found on integrons frequently located on plasmids and transposons (Heuer, Schmitt and Smalla 2011a), which might be transferred from manure bacteria to other soil bacteria and pathogens following manure application (Binh et al. 2008; Allen et al. 2010). In addition, manuring was shown to even increase the permissiveness of the soil community for receiving and maintaining broad-host-range plasmids (Jechalke et al. 2014b). Integrons possess a site specific recombination system that could capture and express mobile gene cassettes (Heuer, Schmitt and Smalla 2011a), and they were reported to often localize in broad-host-range IncP-1ε plasmids or other mobility elements (Heuer et al. 2012; Jechalke et al. 2014c; Wolters et al. 2015). The class 1 integrons are also important for co-selection and mobilization of other ARGs (except for β-lactamases), especially when selective pressure by antibiotics is present (Jechalke et al. 2014c). However, the rate of horizontal transmission in soil was assumed to be very low, compared with the vertical transmission of ARGs due to growth of resistant microorganisms (Heuer et al. 2011b). ARGs originating from soil indigenous microorganisms might have difficulty in spreading to clinical settings, because these β-lactamases might be located in chromosome rather than mobile elements (Udikovic-Kolic et al. 2014). Recent functional metagenomic studies suggested that soil microbial phylogenetic and taxonomic structure is the primary determinant of ARG profile across various soil types, and no positive correlation was found between the soil ARGs and mobility elements suggesting that the horizontal gene transfer between soil bacteria is very low (Forsberg et al. 2014). In fact, integrons are just one of the many types of mobile genetic elements involved in the adaptation of bacteria (Stalder et al. 2012), and we did not find any positive effect of manure application on the transposase gene tnpA. Therefore, although the acquisition of ARGs by lateral gene transfer is considered as the most probable route of ARGs emergence in
clinical environments (Nesme and Simonet 2014), such mechanism is pending more investigations in soil environments. Despite these findings, enriched ARGs can still enhance the likelihood of transmission into crops grown in manure-treated soil even in the absence of transferability, because ARGs might also reside in some opportunistic human pathogens (Udikovic-Kolic et al. 2014). Our network analysis also revealed that the co-occurring ARGs in each module might reside in the same mobile elements, bacteria or pathogens, which might be transferred together to humans under appropriate selection conditions in the future.

In conclusion, by combining microcosm incubations and high-throughput qPCR arrays, we demonstrated strong impacts of antibiotic-free manure application on the abundance, diversity and mobility potential of a broad spectrum of ARGs in both arable and pasture soils. Although pervasive antibiotic use is the most important factor for global dispersal of ARGs, our results suggested that other mechanisms including the introduction of manure-derived ARGs (which can persist in manure-amended soils for several months) and stimulation of resident soil ARGs by antibiotic-free manure fertilizers, can also accelerate environmental ARG spread. Although the effect of a single manure amendment on ARGs will diminish over time due to gradually decreased nutrient levels and manure-derived ARGs, soil ARGs can accumulate with repeated applications of animal manures with antibiotics (Heuer et al. 2011b). These intensive agricultural practices will increase the likelihood of soil and manure-derived ARGs being moved into the food chain, and a subsequent acquisition of antibiotic resistance in human microbiome via consumption of contaminated crops grown in manure-fertilized soils (Marti et al. 2013). Therefore, future research is desirable to design options and management practices that can minimize dispersal of ARGs from harvested crops in manure-treated soils into the food chain.

SUPPLEMENTARY DATA
Supplementary data are available at FEMSEC online.

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