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RESEARCH ARTICLE

Distribution of antibiotic-resistant bacteria in chicken manure and manure-fertilized vegetables

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Abstract Veterinary manure is an important pollution reservoir of antibiotics and antibiotic-resistant bacteria (ARB). However, little is known of the distribution of ARB in plant endophytic bacteria and the number/types of ARB in chicken manure. In this study, 454-pyrosequencing was used to investigate the distribution and composition of ARBs in chicken manure and fertilized vegetables. The prevalence of ARB in the samples of the chicken manure compost recovered from farms on which amoxicillin, kanamycin, gentamicin, and cephalexin were used was 20.91–65.9 % for ARBs and 8.24–20.63 % simultaneously resistant to two or more antibiotics (multiple antibiotic resistant bacteria (MARB)). Antibiotic-resistant endophytic bacteria were widely detected in celery, pakchoi, and cucumber with the highest rate of resistance to cephalexin. The pyrosequencing indicated that the chicken manure dominantly harbored Firmicutes, Bacteroidetes, Synergistetes, and Proteobacteria and that Bacteroidetes was significantly enhanced in farms utilizing antibiotics. In the total cultivable colonies, 62.58–89.43 % ARBs and 95.29 % MARB were clustered in Bacteroidetes with the dominant species (*Myroides ordoratimimus* and *Spningobacterium* spp., respectively) related to human clinical opportunistic pathogens.

Keywords Antibiotic resistance · Chicken manure · Antibiotic-resistant endophytic bacteria · Multiple antibiotic-resistant bacteria · Vegetable · 454-pyrosequencing

Introduction

Antibiotics are probably the most successful family of drugs developed for improving human health. Besides this fundamental application, antibiotics are used to prevent and treat infections in animals and plants and to promote growth in animal farming (Martinez 2009). In the USA, the annual usage of antibiotics is approximately 22,700 tons, with 13,067 tons administered for animals, agriculture, and aquaculture (Kummerer 2009). In China, 210,000 tons of antibiotics are produced every year, of which 90 % are applied in agriculture (48 %) and medicine (42 %), with the remaining 10 % being exported (Zhou et al. 2011). Most veterinary antibiotics are not completely absorbed and as much as 30–90 % of administered veterinary antibiotics are excreted in the feces (Sarmah et al. 2006). Overall, bioactive concentrations of several antibiotics are typically detected in manure, with maximum levels reaching many milligrams per kilogram (Heuer et al. 2011).

The large-scale application of various antibiotics has driven the global dissemination of antibiotic-resistant bacteria (ARB), particularly “super bacteria” resistant to multiple drugs (Martinez 2009). Veterinary manure is an important reservoir of antibiotics and ARBs, which could significantly increase the selection of ARBs and increased prevalence of resistance genes in manure-fertilized soil (Heuer et al. 2011). Furthermore, antibiotic resistance genes are considered as a pollutant that can be transferred environmentally through horizontal gene transfer (HGT) without relying on their corresponding antibiotics or ARBs (Chee-Sanford et al. 2009). The release of high concentrations of antibiotics and resistance genes in natural ecosystems can impact the structure and the activity of environmental microbial populations, and given that environmental microorganisms are the original source of resistance genes acquired through HGT by human pathogens, these changes are relevant for the future of human health (Martinez 2009). Pig manure has been shown to promote mobilization in

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farmland and to contain plasmids conferring antibiotic resistance (Smalla et al. 2000). Thus, farmland supplied with pig manure slurry is considered to favor conjugal gene transfer. This has raised concern about the possible formation of an environmental reservoir of antibiotic resistance genes in farmland that could transfer resistance back to animals or humans via crops (Sengelov et al. 2003).

Our investigation information indicated that some important human clinical antibiotics such as amoxicillin, kanamycin, gentamicin, and cephalexin were generally used in chicken and pig farms around Xinxiang city in China. However, detailed data on the prevalence and types of ARBs, especially multiple antibiotic-resistant bacteria (MARB), that are resistant to these antibiotics in chicken manure are scant. It is also unclear whether ARB and MARB in manure can be transferred to edible vegetables during soil fertilization. The aim of this investigation is to evaluate if the use of chicken manure slurry as fertilizer has created reservoirs of antibiotic resistance in plants.

Experimental procedures

Investigation on antibiotics application in chicken farms

Eleven chicken farms housing 3,000–10,000 chickens were investigated with respect to antibiotics use from March 2010 to February 2011. The survey contents included types and purpose of antibiotics used, methods, frequency, and dosage of giving medicines, manure production, and treatment.

Samples

Feces and compost samples from young or/and adult chickens were collected from four chicken farms that harbored at least 5,000 chickens, in which three farms fed the chicken with similar types of antibiotics and one fed the chicken with traditional Chinese medicines. One sample was the manure mixture from at least five spots in the same farm. Samples were immediately transported to the lab under sterile conditions. Three grams of fresh samples were put into a flask with 100 mL sterile water and glass beads and shaken at 200 rpm for 3 h. The suspensions were used for microbial analysis.

Samples of fresh and healthy vegetable plants, such as celery, pakchoi, and cucumber, were collected from greenhouse vegetable fields that were fertilized with chicken manure. Two samples of celery (celeries 1 and 2) were collected from greenhouse fields that were fertilized with chicken manure annually and every 2 years, respectively. The latest fertilization time was about 10 months for pakchoi, cucumber, and celery 1 and 18 months previously for celery 2 before sampling. Five-spot sampling method was used, i.e., each type of vegetable was collected from five spots of the field and at least five strains of the whole plant were collected from each spot. The vegetable samples were stored in freshness protection packages at 4 °C.

Enumeration of total cultivable heterotrophic bacteria, ARB, and MARB

Colony-forming units of total cultivable heterotrophic bacteria (TCB), actinomycetes, and fungi were determined by a modified plate dilution technique on meat-peptone agar, Gause's starch agar, and Martin agar, respectively (Carter 1993). The incubation was at 30 °C for 1–2 days for bacteria, 3–6 days for actinomycetes, and 3–5 days for fungi. Enumeration of bacteria resistant to amoxicillin, kanamycin, gentamicin, and cephalexin was conducted under the same conditions on meat-peptone agar supplemented with antibiotic at a final concentration of 50, 100, 32, or 100 $\mu g\ L^{-1}$, respectively, according to the breakpoint values defined by Clinical and Laboratory Standards Institute (2009)). Enumeration of MARB was determined by mixing two, three, or all four of the above antibiotics at each antibiotic's final concentration in meat-peptone agar (Jiang et al. 2011). The number of TCB, ARB, and MARB was determined by the average of the replicate samples from three chicken farms.

Isolation and enumeration of antibiotic-resistant endophytic bacteria in the vegetables

For isolation of endophytic bacteria, fresh vegetables were cleaned with water, air-dried, immersed in 70 % ethanol for 3 min followed by immersion in 0.1 % mercuric chloride solution for 3 min and 70 % ethanol again for 1 min, washed five to seven times using sterile water, and finally dried under a sterile condition (Germuda 1998). To ensure the complete surface disinfection, 0.2 mL of the last wash water was spread on meat-peptone agar and cultivated at 30 °C to check for colony growth (Schulz and Draeger 1993). Three grams of disinfected tissue of root, stem, leaf and fruit from different plant strains was cut into pieces and ground together with quarta sand in a mortar. The ground tissue was mixed with 10 mL sterile water and left for 15 min. Each 100 μL suspension was spread on meat-peptone agar and on various antibiotic agars for cultivation at 30 °C for 1–2 days. Each treatment was replicated five times. The colony-forming units of total cultivable endophytic bacteria (TCEB) and antibiotic-resistant endophytic bacteria (AREB) were enumerated (Lupwayi et al. 2004).

Statistical analysis

A one-way ANOVA was performed to determine significant differences ($p<0.05$ and $p<0.01$) between the prevalence of

ARBs and MARB in different samples. Statistical analysis was performed using the software SPSS 21 (Zhou et al. 2011).

Total DNA extraction of environmental manure samples, TCB, ARB, and MARB

For total bacteria community analysis, manure was washed three times using phosphate-buffered saline (pH 8.0) and centrifuged at 4 °C and 10,000 rpm for 15 min for total genomic DNA extraction. The total genomic DNA of the microbial community was extracted using CTAB (Jia et al. 2006). For TCB, ARB, and MARB analyses, colonies of bacteria growing on antibiotic-free or antibiotic-containing meat-peptone agar were harvested and mixed. The mixture of TCB or total ARB or MARB was respectively cultivated in liquid LB medium for 24 h for total genomic DNA extraction. Total DNA was extracted according to a standard method (Stahl et al. 1988).

High-throughout 454-pyrosequencing

The composition of the polymerase chain reaction products of the V3 region of the 16S rRNA gene was determined by 454-pyrosequencing by BGI (Shenzhen, China). The bacterial universal primer pair, 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 534R (5′-ATTACCGCGGCTGCTGG-3′) were used (Guo et al. 2012). Samples in this study were individually barcoded to enable multiplex sequencing. Following pyrosequencing, Python scripts were written to remove: (1) sequences containing more than one ambiguous base, (2) check the completeness of the barcodes and the adapter, and (3) remove sequences shorter than 150 bps (Ye et al. 2011). The effective sequences were analyzed using Ribosomal Database Project (RDP) to construct the distance matrices, assign sequences to operational taxonomic units (OTUs; 97 % similarity), and calculate Chao1 richness estimators (Schloss et al. 2009). Sequences of the dominant OUTs were extracted to run BLAST and search relatives against "nr" database through internet automatically (http://www.ncbi.nlm.nih.gov/BLAST/). A phylogenetic tree was constructed using the neighbor-joining method in MEGA version 4.1 using 1,000 bootstrap replications. The archaeon *Methanobacterium formicicum* was used as an out-group.

Results and discussion

Survey on antibiotic application in chicken farming process

Before detecting the distribution of ARBs, 11 chicken farms harboring 3,000–10,000 chickens were surveyed with respect to their use of antibiotics. Ten farms fed chickens with at least four different types of human clinically relevant antibiotics (amoxicillin, kanamycin, gentamicin, and cephalexin). The remaining farm used traditional Chinese medicines (mixture 1: *Pulsatilla chinensis*, 60 g; *Coptis chinensis* Franch, 30 g; Cortex Phellodendri Chinensis, 45 g; Cortex Fraxini, 60 g; mixture 2: *Andrographis paniculata*, *Radix Glycyrrhizae*, *Tetradium ruticarpum*, Radix Sophorae Flavescentis, Radix Angelicae Dahuricae). In the ten farms, one or more antibiotics were added to the drinking water or feedstuffs of young chickens under 50 days of age to prevent disease and/or promote growth. The feeding dosages were typically markedly higher than the limits of the drug dosage. For adult chickens more than 50 days of age, the various antibiotics were normally used only to prevent/treat disease and were applied as described above. The traditional Chinese medicines were added in the feedstuffs at the dosage of 1 kg medicine mixture 1 or 2/500 kg feedstuffs to prevent disease. Based on the farm size, 300–4,000 kg fresh manure was produced everyday. Most of the manure was directly applied on vegetable fields as fertilizer after being composted for about 1 week.

Distribution of ARB in chicken manure

Three farms (harboring 5,000–8,000 chickens) that fed antibiotics and one farm that fed Chinese medicines were selected to investigate ARBs distribution.

The viable number of heterotrophic bacteria (TCB), actinomycetes, and fungi in chicken manure composted for 1 week was 2.37×10^{12}–3.91×10^{12}, 2.03×10^{10}–1.85×10^{11}, and 1.72×10^{7}–7.77×10^{11} CFU g^{-1}, respectively. Bacteria were the predominant population. ARBs were mainly detected in the chicken manure compost, especially in the farms engaged in the use of antibiotics, in which the viable count of ARBs resistant to any one antibiotic and MARB resistant to two or more was 2.65×10^{11}–4.01×10^{12} and 1.44×10^{10}–7.60×10^{11} CFU g^{-1}, respectively. In the farm utilizing Chinese medicines, ARBs were also generally detected, but at much lower levels, ranging at 4.90×10^{9}–3.50×10^{11} CFU g^{-1} of ARBs and 3.34×10^{8}–5.48×10^{10} CFU g^{-1} of MARBs.

Figure 1 presents the prevalence of ARBs and TCB in the chicken manure compost and fresh manures of young and adult chickens. ARB that was resistant to cephalexin was the most prevalent in all the investigated farms. The rates of various ARBs and MARBs in the chicken manure compost that utilized antibiotics were 20.91–65.9 and 8.24–20.63 %, respectively. These rates were markedly greater than the rates of the farm that used Chinese medicines (0.64–26.87 % for ARBs and 0.02–4.13 % for MARB in the manure compost; $p<0.05$), and also higher than fresh chicken manure samples (6.88–18.51 % ARBs and 3.0–25.2 % MARB for young chickens and 10.15–33.17 % ARBs and 4.45–13.71 % MARB for adult chickens). Although the rates of various

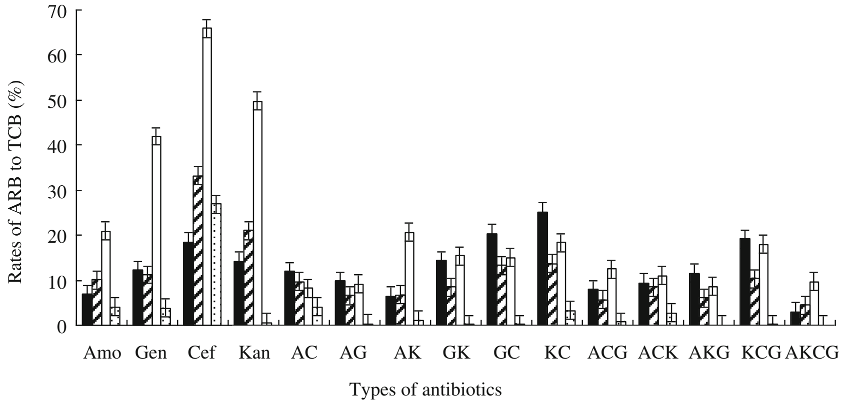

Fig. 1 Rates of ARB to total cultivable bacteria (*TCB*) in chicken manure (*Amo* amoxicillin, *Kan* kanamycin, *Cef* cephalexin, *Gen* gentamycin. AC to AKCG represents different combination of the four types of antibiotics. ■ fresh manure of young chicken from antibiotic-feeding farms; ▨ fresh manure of adult chicken from antibiotic-feeding farm; □ manure compost from antibiotic-feeding farms; ⬚ manure compost from the Chinese medicines feeding farm. The values were the average of three samples collected from three chicken farms. Difference between the manure compost samples of antibiotic-feeding farms and the farm supplying Chinese medicines is $p<0.05$. Difference between cephalexin resistance and resistance to other antibiotics is $p<0.05$)

ARBs in the young chicken manure were not comparable to the data in the adult chicken manure, the prevalence of MARB in the former were generally higher than those in the latter samples ($p<0.05$).

The previous and present results indicate that feeding with antibiotics, especially more than one, significantly favors the growth of ARBs and MARBs in animal manure. However, the prevalence of resistance bacteria present in animal manure seemed to be correlated with animal species and the relative amounts of antibiotics used; for example, the frequency of bacteria carrying antimicrobial resistance genes was reported to be especially high for pigs as compared with cattle or sheep and correlated with the amounts of antibiotics used in the husbandry of these animal species (Enne et al. 2008; McKinney et al. 2010). Presently, the viable concentrations of various ARBs and MARBs in chicken manure samples were significantly higher than the levels in pig manure compost (4.21–49.12 % of ARBs and 0.07–3.22 % of MARB; $p<0.05$ between chicken and pig manure). This was very likely related to the specified prescription of antibiotics application in different animals. Based on our survey, similar types of antibiotics were used for pigs and chicken but with lower frequency and lower relative dosage in pig farms. Furthermore, feeding chicken with the traditional Chinese medicines instead of antibiotics significantly decreased the occurrence of ARBs and MARB in the manure.

The higher prevalence of MARB presence in the young chicken manure was also likely related to the fact of higher dosages and more than one type of antibiotics used for young chicken. Studies involving pigs (Thompson et al. 2008) and newly weaned calves (Peak et al. 2007) reported that bacterial populations established in gut very early in the life of piglets from their immediate environment, and the common practice of antibiotic treatment as a prophylaxis against postweaning diarrhea favors the establishment of resistance bacteria in the gut microflora.

However, the higher occurrence of ARBs and MARB in chicken manure compost (after one-week composted) was possibly related to three mechanisms: (1) mutation of the indigenous bacteria in the manure (Costanzo et al. 2005), (2) advantaged growth of intrinsically resistant bacteria or mutants under the selective pressure of antibiotics that were discharged with manure (Martinez 2009), and (3) development and spread of resistance through HGT among diverse microorganisms (Costanzo et al. 2005). If antibiotic resistance genes, already located in gene-transfer units, are present as pollutants in the manure, the presence of antibiotics and nutrients in the chicken manure will favor their dissemination and thus contribute to the high prevalence of ARBs and MARB (Chee-sanford et al. 2009, Martinez 2009, Knapp et al. 2010, Sengelov et al. 2003). Although we did not detect the antibiotic compounds in the chicken manure in this study, a prior study reported that 30–90 % of the applied veterinary antibiotics could not be absorbed by animals and were discharged unaltered in feces, with concentrations in manure reaching many milligrams per kilogram (Heuer et al. 2011).

Cephalexin is the first-line human prescription drug inhibiting both Gram-positive and Gram-negative bacterial pathogens. There is little information on the distribution of cephalexin in soil or animal manure. However, cephalexin was the most frequently detected antibiotic and in highest concentrations in sewage influents reaching 2.0–4.6 $\mu g\ L^{-1}$ in Australia (Costanzo et al. 2005, Watkinson et al. 2007). This drug did demonstrate persistence in receiving waters due to its detection at 500 m from the source (Watkinson et al. 2007). Based on our survey on antibiotics application, cephalexin was applied in all the chicken farms that used antibiotics as feed additives and generally in dosages over the limit of the drug dosage. However, different from other antibiotics, cephalexin resistance occurred in high prevalence in all the samples including the farm supplying with Chinese medicines. This suggested that high prevalence of intrinsic cephalexin resistance existed in the chicken manure. During 1-week preparation of the manure compost, the intrinsic cephalexin-resistant bacteria were enhanced under the selective pressure of antibiotics exposure, especially in antibiotics supplying farms, as indicated by the higher prevalence of cephalexin-resistant bacteria in chicken compost (Fig. 1). They will reach soil through manure slurry. Although intrinsic resistance is normally encoded in the non-mobile (chromosomal) genetic pool, even such genes might occasionally be transferred to the native bacterial population and become the potential to create a widespread reservoir of cephalexin resistance as stressed by Costanzo (Costanzo et al. 2005).

Distribution of AREB in chicken manure fertilized vegetables

Presently, chicken manure was used directly as fertilizer for vegetables after being composted for 1 week. To clarify whether ARBs and MARBs in the manure were retained on or in the fertilized vegetables, celery, cucumber, and pakchoi were examined to investigate possible distribution of AREB in rhizome, fruit, and leaf vegetables that are routinely consumed (Table 1).

No AREB resistant to two or more antibiotics was detected, suggesting the absence of MARB on or in the tissues of vegetables. However, high numbers of AREB resistant to one antibiotic were evident in all vegetables examined. This was especially the case for bacteria resistant to cephalexin, with the prevalence of AREB as the percentage of TCEB being 16.9–86.35, 21.76–91.32, and 0.21–0.44 % in different parts of celery, pakchoi, and cucumber, respectively, consistent with the results in chicken manure compost. The results further supported the above idea that resistance to cephalexin was selected most readily among the four antibiotics examined ($p<0.05$ between cephalexin and amoxicillin/kanamycin/gentamicin), and the speculation that cephalexin resistance transferred from soil to plants. The rates of kanamycin-resistant AREB were significantly lower than those for other antibiotics and also lower than the results in fresh chicken manure or compost.

Although the total numbers of AREBS for kanamycin and cephalexin were higher in celery 2 (the celery that was fertilized with chicken manure every 2 years), the rates of AREBs in celery 1 (celery that fertilized annually) were generally higher (1.7 to 1715 times) than those in the celery 2, especially in roots ($p<0.05$) and stems ($p<0.05$), suggesting that the fertilization process and frequency of chicken manure application might influence the detection rates of AREB in celery. There is scant information on the persistence of these four types of antibiotics and the corresponding ARB in fertilized soil. The report from Sengelov et al. indicated that the occurrence of tetracycline-resistant bacteria was elevated after spread of pig manure slurry and higher load of pig manure slurry yielded higher occurrence of tetracycline resistance after spreading but declined throughout the sampling period (5 months) to a level corresponding to the control soil (Sengelov et al. 2003). Furthermore, Schmitt et al. studied the diversity of tetracycline-resistant genes as influenced by

Table 1 Concentrations of AREB and rates of AREB to TCEB in different parts of chicken manure fertilized greenhouse vegetables (in colony-forming units per gram)

Vegetables		TCEB (×10^9)	Amo^r		Kan^r		Ceph^r		Gen^r	
			AREB (×10^7)	Rates (%)	AREB (×10^7)	Rates (%)	AREB (×10^7)	Rates (%)	AREB (×10^7)	Rates (%)
Celery 1 (fertilized every year)	Root	2.26±0.77	70.6±0.55	31.24	18.8±0.5	8.32	139±1.46	61.50	73±0.85	32.30
	Stem	5.22±0.9	113±1.21	21.65	0.97±0.23	0.19	88.2±0.76	16.90	0.025±0.001	0
	Leaf	1.04±0.66	16.9±0.83	16.25	0.95±0.67	0.91	89.8±0.89	86.35	0.4±0.01	0.38
Celery 2 (fertilized every two years)	Root	7.48±0.32	28.1±0.45	3.76	139±1.13	18.58	236±2.99	31.55	0.138±0.001	0.02
	Stem	4.51±0.27	35.2±0.55	7.80	31.2±0.44	6.92	44.6±0.1	9.89	0.075±0.26	0.02
	Leaf	1.35±0.21	23.5±0.41	17.41	3.41±0.04	2.53	101±0.74	74.81	0.356±0.01	0.26
Cucumber	Root	0.66±0.01	0.01±0.005	0.01	0.0006±0.0001	0	0.22±0.01	0.33	0	0
	Stem	0.17±0.01	0.082±0.008	0.48	0.0003±0.0001	0	0.075±0.01	0.44	0	0
	Leaf	0.31±0.04	0.002±0.001	0.01	0.0022±0.0001	0.01	0.065±0.001	0.21	0	0
	Fruit	0.51±0.08	0.011±0.002	0.02	0.018±0.0002	0.04	0.11±0.03	0.21	0	0
Pakchoi	Root	0.71±0.43	13.7±1.1	19.27	0.037±0.002	0.05	42.9±5.69	60.34	21.5±4.57	30.24
	Stem	3.12±0.16	36.9±3.27	11.83	0.005±0.001	0	67.9±3.85	21.76	18.4±3.29	5.90
	Leaf	4.03±0.28	194±5.63	48.14	0.083±0.011	0.02	368±2.89	91.32	384±3.64	95.29

The values in the table are average of five samples replicates from one field. The tissues mixture of 5–10 strains of plants constituted each sample

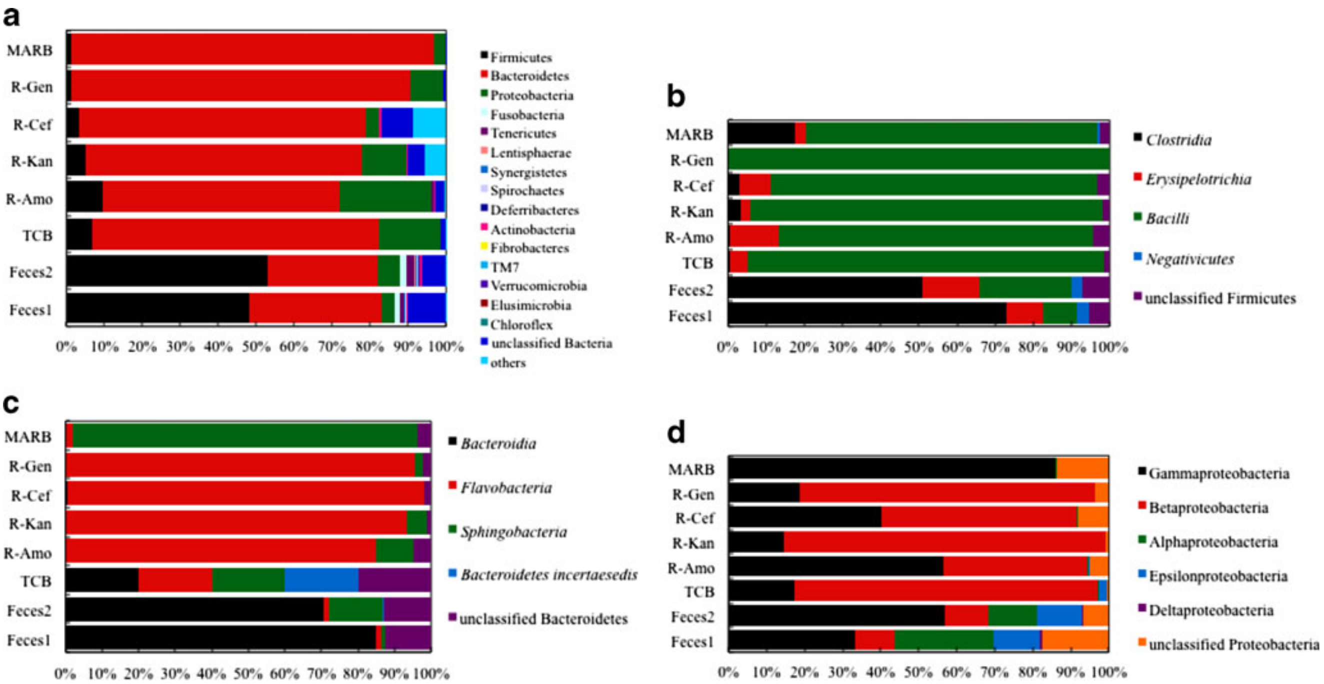

Fig. 2 Bacterial and ARB compositions in chicken feces based on RDP analysis of 16S rRNA gene sequences detected by 454-pyrosequencing method (**a** total composition, **b** compositions of Firmicutes, **c** compositions of Bacteroidetes, and **d** compositions of Proteobacteria; feces 1 and 2, from chicken farms feeding with antibiotics and Chinese medicines, respectively; *TCB* total cultivable bacteria in feces 1, *R-Amo* bacteria resistant to amoxicillin, *R-Kan* bacteria resistant to kanamycin, *R-Cef* bacteria resistant to cephalexin, *R-Gen* bacteria resistant to gentamycin—including bacteria which were resistant to more than one antibiotics, *MARB* multiple antibiotic-resistant bacteria)

fertilization with pig manure in soil microcosms. They found that both manure and soil contained a high diversity of resistance genes, however, the influence of fertilization with manure was clearly shown by an increase in the abundance of resistance genes in the soil after manuring (Schmitt et al. 2006). Therefore, although the mechanisms of transfer of antibiotic resistance from manure-amended soil to plants is not clear, the high occurrence of AREB in celery fertilized every year is likely related to the high input of ARBs that might carry antibiotic resistance genes and high load of antibiotics in the soil due to increased application of manure. However, the influence of manure fertilization frequency on occurrence of AREB is required to be further confirmed by more investigation.

In most plants, roots generally contain the highest rates of TCEB and AREB. Thus, the roots might be a reservoir for bacteria and ARBs (Berg et al. 2005). In our previous report about ARBs in wheat, AREBs were most prevalent in roots followed by stems followed by leaves, with no AREBs being detected in seeds (Yang et al. 2009). In this study, similar results were evident only in celery. There was no significant difference of AREB distribution in different tissues of cucumber ($p>0.05$) while obviously higher rates of AREBs were detected in the leaves of pakchoi. Cucumber contained the lowest numbers of AREBs generally two orders of magnitude lower than the other vegetables ($p<0.05$).

The paucity of information about plant AREB hinders knowledge of how AREBs are produced. In one study, corn (*Zea mays* L.), lettuce (*Lactuca sativa* L.), and potato (*Solanum tuberosum* L.) could take up the sulfonamide-class antibiotic, sulfamethazine, from manure-amended soil with concentrations in plant tissue ranging from 0.1 to 1.2 mg kg^{-1} dry weight (Dolliver et al. 2007). The uptake of antibiotics differs among plants and with different types of antibiotics. In general, the concentrations of antibiotics in plant tissues increases with increasing amounts of antibiotics present in manure (Kumar et al. 2005; Dolliver et al. 2007). Therefore, it is reasonable to suppose that subinhibitory concentrations of antibiotics in plant tissues are a driver of AREB production. However, as addressed above, the high prevalence of ARBs that carried resistance genes in manure could enter amended soils through fertilization process and influence the numbers of ARBs and resistance genes in soil ecosystem. It is still unclear whether ARBs can directly enter plant tissues and whether the resistance genes in manure-amended soil can be taken up directly by plants.

Fig. 3 Phylogenetic tree (**a**, **b**) of ARB and MARB compositions in ▶ chicken feces based on RDP analysis of 16S rRNA gene sequences detected by 454-pyrosequencing method (*empty circles* total cultivable bacteria (*TCB*), *filled squares* bacteria resistant to amoxicillin, *filled triangles* bacteria resistant to kanamycin, *empty triangles* bacteria resistant to cephalexin, *empty squares* bacteria resistant to gentamycin, *filled circles* multiple antibiotic-resistant bacteria (*MARB*); operational taxonomic units of >50 were selected for construction of the tree)


a
55
Proteobacteria
63
Firmicutes
99
○ TCB3717
100
AB264628.1 ***Bacteroidaceae bacterium***
△ R-Cef3717
AB542765.1 ***Bacteroides propionicifaciens***
Bacteroidetes
HE800567.1 ***Sphingobacterium daejeonense***
▲ R-Kan2600
● MARB3087
98
● MARB4835 1
□ R-Gen3087
99
JF708890.1 ***Sphingobacterium mizutaii***
75
■ R-Amo3087
■ R-Amo9924
77
▲ R-Kan9924
86
▲ R-Kan5921
59
▲ R-Kan4608
73
NR 040953.1 ***Sphingobacterium multivorum***
Sphingobacteria
85
88
○ TCB10272
△ R-Cef10272
□ R-Gen5903
65
NR 044698.1 ***Myroides odoratus***
▲ R-Kan5903
100
△ R-Cef4760
86
△ R-Cef6644
○ TCB4760
■ R-Amo7412
54
○ TCB7412
JQ229805.1 ***Myroides odoratimimus***
△ R-Cef7412
■ R-Amo7869
△ R-Cef7832
▲ R-Kan988
40
△ R-Cef5841
△ R-Cef6410
□ R-Gen6410
○ TCB8115
□ R-Gen7412
△ R-Cef8033
○ TCB7869
○ TCB5841
▲ R-Kan8115
25
▲ R-Kan5445
△ R-Cef7869
○ TCB6410
△ R-Cef2666
■ R-Amo5841
▲ R-Kan5841
■ R-Amo8115
▲ R-Kan6410
▲ R-Kan7869
▲ R-Kan7412
■ R-Amo6410
△ R-Cef8115
● MARB7412
○ TCB8337
○ TCB4419
61
△ R-Cef4419
Flavobacteria
Bacteroidetes
M36508.1 ***Methanobacterium formicicum***
0.05

Fig. 3 (continued)

Analysis of bacterial community structure of TCB, ARB, MARB, and chicken manure

Clones of TCB, ARB isolates, and MARB were harvested from antibiotic-free or antibiotic-containing agars for 454-pyrosequencing analysis together with the chicken manure samples acquired from farms using antibiotics and Chinese medicines. A total of 7,088 and 13,301 sequences were obtained for the environmental samples from the antibiotic-feeding farms and the Chinese medicine-feeding farm, respectively, and total of 7,750, 24,533, and 12,048 sequences obtained for TCB, ARB, and MARB, respectively. From the sequences of TCB, ARB, and MARB, 95 OTUs were obtained based on RDP analysis. Figure 2 summarizes the bacterial compositions of the manure samples, TCB, ARBs, and MARB, while Fig. 3 shows the detailed phylogenetic compositions of TCB, ARBs, and MARB.

The dominant bacterial populations were very similar in the two chicken manure samples acquired from farms utilizing antibiotics and Chinese medicines farms (Fig. 2). Four major phylums (Firmicutes, Bacteroidetes, Synergistetes, and Proteobacteria) were detected in the manure samples with Firmicutes the predominant population occupying 48.05 and 53.06 % of the total in antibiotic-containing and antibiotic-free manures, respectively (Fig. 2a). Firmicutes was mainly composed of Clostridia, Erysipelotrichia, Bacilli, Negativicutes, and some unclassified Firmicutes, in which Clostridia was the predominant population (Fig. 2b). Bacteroidetes was significantly enhanced comparing with other phyla in the manure samples of farms feeding with antibiotics, and its compositions were different from those in the manure sample of the farm supplying with Chinese medicines. Figure 2c indicated that Bacteroidetes were mainly composed of Bacteroidia, Sphingobacteria, Flavobacteria, and unclassified Bacteroides, in which Sphingobacteria was significantly inhibited and Bacteroidia was enhanced in the antibiotic-containing manure samples. Different from the report by Li (Li et al. 2011), although Proteobacteria were generally detected in all the samples, they were not the most dominant population whether or not under the selective pressure of antibiotics. Their compositions are indicated in Fig. 2d. Gamma-, Beta-, Alpha-, Epsilon-, and Deltaproteobacteria were present in both the chicken manure samples with Gammaproteobacteria being the predominant population although the ratios were different among different populations.

Although Firmicutes was the dominant population in chicken manure samples, Bacteroidetes constituted 75.37 % of the TCB, followed by Proteobacteria and Firmicutes. In the various cultivable ARBs, Bacteroidetes was again the predominant phylum (62.58 to 89.43 % of the total ARBs). ARBs resistant to cephalexin and kanamycin had the most diverse bacterial compositions. The diversity of MARBs was the lowest, constituting only three phyla with Bacteroidetes occupying 95.29 % of the total. This is probably due to the fact that Bacteroidetes are intrinsically resistant to various antibiotics.

Although Bacteroidetes constituted the predominant population of TCB, ARBs and MARB, various antibiotics significantly inhibited most genera of Bacteroidetes, resulting in absolute dominance of Flavobacteria in various ARBs. Most OTUs of various ARBs were clustered together with *Myroides ordoratimimus* of Flavobacteria (Fig. 3). *Myroides* spp. is yellow-pigmented, non-fermenting Gram-negative rods that are resistant to multiple antibiotics. *Myroides* spp. behaves like low-grade opportunistic pathogens and has been identified as a source of surgery wounds and urinary tract infections, septicemia, pneumonia, meningitides, fasciitis, and ventriculitis. *Myroides odoratimimus* strains are responsible for cases of urinary infection and one case of cellulitis associated with bacteraemia (Ktaria et al. 2012).

However, Flavobacteria was not the best host bacterium for MARB. MARB mainly involved Sphingobacteria, which was composed of some unidentified species of genus *Sphingobacterium* as revealed in Fig. 3. Bacteria that belong to genus *Sphingobacterium* are Gram-negative, non-fermentative microorganisms and contain high concentrations of sphingophospholipids in the cellular lipid components. The genus *Sphingobacterium* consists of eight species: *Sphingobacterium spiritovorum*, *Sphingobacterium multivorum*, *Sphingobacterium mizutaii*, *Sphingobacterium antarcticum*, *Sphingobacterium faecium*, *Sphingobacterium piscium*, *Sphingobacterium heparinum*, and *Sphingobacterium thalpophilum*, among which *S. multivorum* and *S. spiritovorum* have been associated with bacteremia, peritonitis, and chronic respiratory infection in patients with severe underlying conditions (Krieg 2010). Furthermore, all the isolates of *S. multivorum* from the patients with respiratory infection showed multidrug resistance, while *S. spiritovorum* were not considered multiresistant (Lambiase et al. 2009).

Other identified ARBs and MARB in this study included *Escherichia coli*, *Aeromonas media*, *Proteus mivabilis*, *Bacteroides propionicifaciens*, *Enterococcus* spp., *Bacillus* spp., *Sporosarcina* spp., *Comamonas* spp., *Alcaligenes* spp., *Proteus* spp., and some unidentified Bacilli and Proteobacteria. *E. coli* was largely reported as a bioindicator of antimicrobial resistance (Jiang et al. 2011; Delsol et al. 2010). However, in this study, although *E. coli* was found in ARBs resistant to kanamycin and amoxicillin, it was not the predominant ARB or MARB in chicken manure. Both the dominant ARB identified as *M. ordoratimimus* and the dominant MARB identified as *Sphigobacterium* spp. were clinical multiresistant bacteria and were related to various disease infections. Therefore, their distribution in environments and risks on human health are worthy of concern.

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