· 论著 ·

家养及大型养殖场鸡肠道微生物菌群及四环素耐药菌多样性的比较研究

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摘　要：本研究分析比较了农家家养鸡与大型养殖场鸡肠道菌群组成及抗四环素抗性基因在肠道菌群中的分布情况。通过 454 焦磷酸法对细菌 16S rRNA V3 区进行测序来分析肠道菌群的组成；用平板琼脂法筛选四环素耐药菌株，对其 16S rRNA 基因全长测序，并与 RDP（Ribosomal Database Project）数据库比对进行菌种鉴定；聚合酶链反应（polymerase chain reaction，PCR）检测常见四环素耐药基因。家养鸡粪便中菌群香农多样性指数为 5.321 ± 0.590，养殖场鸡为 4.398 ± 0.440，前者显著大于后者（Mann-Whitney *U* test，P = 0.008）。从家养鸡和养殖场鸡粪便中随机分离到 69 株和 65 株四环素耐药菌株，后者四环素耐药菌的种类多于前者。家养鸡较养殖场鸡的肠道菌群更具多样性，而抗生素抗性基因在养殖场鸡的肠道菌群中分布更广泛。结果表明，不同饲养方式对鸡的肠道菌群有影响，对抗生素抗性基因的分布也有一定影响。

关键词：鸡粪；微生物群；四环素耐药；多样性

Comparison of microbiota and dissemination of tetracycline resistant bacteria between chickens from a small farmhouse and a big feedlot

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Abstract: To analyze the microbial composition and the distribution of tetracycline resistant bacteria in the feces of chickens from small farmhouse and big feedlot, eight chickens from a small farmhouse and ten chickens from a big feedlot were enrolled. Fecal microbiota was analyzed through 454 pyrosequencing of 16S

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rRNA V3 region. Fourteen reported tetracycline resistance genes were tested by polymerase chain reaction (PCR). The mean Shannon diversity indexes of microbiota in chickens from a small farmhouse and a big feedlot were 5.321 ± 0.590 and 4.398 ± 0.440, respectively, with significant difference (Mann-Whitney *U* test, $P = 0.008$). Moreover, 69 and 65 tetracycline resistant isolates were randomly selected from chicken feces in small farmhouse and big feedlot, respectively. The species of tetracycline resistant bacteria isolated from chicken feces in the big feedlot were more diverse than those in the small farmhouse. The data concluded the wider dissemination of tetracycline resistant bacteria in the chickens from big feedlot, suggesting that more attentions should be paid to the changes in the microbiota and tetracycline resistant bacteria in the chickens from big feedlot and their influences on human health.

Key words: Chicken feces; Microbiota; Tetracycline resistance; Diversity

Antibiotics as growth promoters have been widely used in agricultural animals for more than 50 years due to the beneficial effect on improving feed conversion, speeding animal growth and reducing diseases[1]. However, the role of antibiotic growth promoters (AGPs) in the emergence of antibiotic resistance has been documented. One of the first reports of antibiotic resistance was published in 1951 after experimental feeding of streptomycin in turkeys[2]. Pathogenic bacteria resistant to multi-antimicrobial agents emerged worldwide in the 1980s and are becoming a major public health concern[1]. Although many factors are involved in the emergence of resistant strains, routine use of antibiotics at sub-therapeutic level in animal feeding is a major risk in driving the emergence of antimicrobial resistance. The intestinal microbiota was composed of combination of complex bacterial populations. The microbiota will be affected by environmental factors, especially antibiotics. Antimicrobial resistant bacteria are able to counteract with antibiotic treatment which will lead them to be the predominant groups in the microbiota. Concerns have been raised to the use of antibiotics as growth promoters in livestock due to their side effect. European Union has banned nonessential antibiotics as growth promoters in food animals since January 1, 2006. US Food and Drug Administration (FDA) also took steps to reduce the use of AGPs in March, 2012. Chinese Ministry of Agriculture announced a forthcoming ban on antibiotics as growth promoters in animal feeding in 2011.

There have been many reports focusing on contamination by antibiotic resistant bacteria detected in chicken meat. Gastrointestinal tract is a massive reservoir of bacteria with a potential for both receiving and transferring antibiotic resistance genes[3]. Most of resistance genes identified in microflora using culture-independent sampling have not been reported before and are evolutionarily distant from the known resistance genes[4].

Chicken feces are important in pathogen transmission to human food chain and then further communicating with human microbiota[5-6]. In this study, the fecal microbiota and tetracycline resistant bacteria in chickens from a small farmhouse and a big feedlot were analyzed to identify the changes in microbial composition and the distribution of tetracycline resistant bacteria.

1 MATERIALS AND METHODS

1.1 Ethics statement

No specific permissions were required for the described field studies, as well as for the chicken feces in Liyang City, Jiangsu Province, China.

They were permitted by their hosts. The field studies did not involve any endangered or protected species. This study has been reviewed and approved by the Ethics Committee of Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine.

1.2 Sampling

A group of 8 chickens raised in a small farm-house (totally 8 chickens) were recruited from Licheng Town, Liyang City, Jiangsu Province, China. All chickens were fed with cetotetrine hydrochloride for 13 d before sampling because of illness. The age of chickens was 37 d and the average weight was 125 g. The diets of chickens included rice bran, Chinese cabbage and cornmeal mush. Another group of 10 chickens raised in a big feedlot (totally 10 000 chickens) were recruited from Nandu Town, Liyang City, Jiangsu Province, China. The age of chickens was 35 d and the average weight was 1 450 g. The chickens were fed with penicillin (1-2 u/kg) for 3 d and amplidox (feed/amplidox 200 g/g) for 5 d (once per day). On the 10th and 15th days, the chickens were vaccinated with attenuated Newcastle disease and influenza vaccines separately. The diets of the chickens from feedlot were purchased from Nantong Chia Tai Co., Ltd. Individual fresh feces of each chicken was collected from the portions of the droppings to avoid any contamination. The collected feces were transported on ice to Shanghai Jiao Tong University immediately for further experiment.

1.3 Selection and identification of tetracycline resistant clones

The appropriate amounts of feces were taken from each collected sample and diluted ten times in series. A series of fecal dilution in 0.9% NaCl buffer were cultured on Mueller Hinton (MH) plates containing 16 μg/mL tetracycline (Sigma) at 37 ℃ for 24 h in aerobic condition. Five to ten colonies were randomly selected from each sample and cultured in MH broth containing 16 μg/mL tetracycline. Genomic DNA of each isolate was extracted using Bacterial DNA Extraction Kit according to the protocol provided by the manufacturer (Omega, USA).

Each tetracycline resistant isolate was identified by 16S rDNA sequence analysis. Genomic DNA of each isolate was used for polymerase chain reaction (PCR) of 16S rRNA gene with the forward primer of 27f: 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse primer of 1492r: 5′-GGTTACCTTGTTACGACTT-3′. The PCR reactions were performed as follows: 95 ℃ for 5 min, 30 cycles of 95 ℃ for 30 s, 55 ℃ for 45 s, 72 ℃ for 2 min, and final extension at 72 ℃ for 10 min. The PCR products were sequenced by Shanghai Majorbio Bio-pharm Technology Co., Ltd (Shanghai, China) and further blasted with National Center for Biotechnology Information (NCBI) database. Sequences displaying greater than 97% identity were assigned to the same species, and sequences displaying greater than 95% identity were assigned to the same genus.

For the analysis of tetracycline resistance genes, the most common tetracycline resistance genes including *tetA*, *tetB*, *tetC*, *tetD*, *tetE*, *tetG*, *tetK*, *tetL*, *tetM*, *tetO*, *tetS*, *tetAP*, *tetQ*, and *tetX* were tested. Genomic DNA of each isolate was used for PCR with each tetracycline resistance gene primer as described[7]. The PCR products were analyzed on a 1.2% agarose gel.

1.4 PCR enrichment of 16S rDNA V3 region and establishment of pyrosequencing library

Each fecal sample was homogenized individually in the liquid nitrogen and kept cool during the procedure. The whole genomic DNA of each fecal sample was extracted using QIAamp DNA Stool Mini Kit (Qiagen) according to the protocol provided by the manufacturer. PCR

enrichment of the 16S rRNA V3 hyper-variable region was performed with the forward primer 5′-XXXXXXXX-TACGGGAGGCAGCAG-3′ and the reverse primer 5′-XXXXXXXX-ATTACCG-CGGCTGCTGG-3′ with the whole genomic DNA directly extracted from feces as templates. The 5′ terminus of each primer contained a different 8-base-oligonucleotide tag (represented by "XXXXXXXX" in the primer sequence), while the sequence after the hyphen was used to amplify the sequence of V3 end region. To ensure a sufficient quantity of PCR product, the 16S rDNA V3 hyper-variable region of PCR products was amplified by two-step PCR strategy as detailed elsewhere[8-9]. The enriched PCR products were mixed equally to establish the library with 50 ng per feces and further sequenced with Roche 454 FLX platform by ZhongXin Biotechology (Shanghai, China).

1.5 Phylogenetic and comparative analysis

The gross sequencing data output by Roche 454 FLX platform were arranged by primer tags. The sequences were first searched for the linker, primers, and their reverse complements using the platform software the company provided. The identified primer sequences we used were trimmed from each sequence read. Sequence reads that did not contain the 5′-end primer were removed from the dataset. The same program was also used for barcode identification. Barcodes were identified within the first 25 bases of the reads. The clean sequences were stored as SRA accession SRA053698.

The datasets were taxonomically grouped using the Ribosomal Database Project (RDP) classifier at a confidence level of 90%[10]. Individual sequences were aligned using the Aligner tool. Aligned sequences for each chicken were processed by complete-linkage clustering using distance criteria. Uclust was used to cluster all the sequences and the value of cut-off was 97%. After clustering, we used the representation sequence of each kind as OUT (http://drive5.com/usearch/manual/otu_clustering.html), and records of each OUT sequence represented the number of sequences and the information of classification. These data were used to calculate the Shannon diversity. UniFrac software was used to analyze the phylogenetic microbial communities of two kinds of samples[11]. SPSS 19.0 was used for statistical analysis and the heat map was drawn by R language (http://www.r-project.org/).

2 RESULTS

2.1 Identification of tetracycline resistant isolates and distribution of tetracycline resistance genes

Sixty-nine tetracycline resistant clones were isolated from feces of chickens raised in a small farmhouse which belonged to 8 species based on 16S rRNA sequence classification, while sixty-five tetracycline resistant clones were isolated from feces of chickens raised in a big feedlot which belonged to 19 species (Tab. 1). The results showed that diversity of antibiotic resistant bacteria in feces of chickens from big feedlot was more than that from farmhouse. The genera including *Escherichia coli* and *Enterococcus* spp. were the major antibiotic resistant carrier in both groups (Tab. 1), whereas *Enterococcus faecium* was only found in the feces of chickens from farmhouse and *Enterococcus casseliflavus* and *Enterococcus gallinarum* only found in the feces of chickens from big feedlot. The antibiotic resistant strains including *Acinetobacter johnsonii*, *Enterococcus faecium*, *Sporosarcina aquimarina*, *Staphylococcus arlettae*, and *Staphylococcus chromogenes* were only presented in feces of chickens from small farmhouse, while the antibiotic resistant strains including *Acinetobacter lwoffii*, *Brachybacterium alimentarium*, *Brachybacterium conglomeratum*, *Brachybacterium* sp., *Corynebacterium* sp., *Corynebacterium stationis*, *En-*

terococcus casseliflavus, *Enterococcus gallinarum*, *Jeotgalicoccus halophilus*, *Pseudomonas putida*, *Psychrobacter pulmonis*, *Raoultella ornithinolytica*, *Shigella* sp., *Staphylococcus equorum*, *Staphylococcus sciuri* and *Staphylococcus* sp. were only found in the feces of chickens from big feedlot (Tab.1).

Fourteen reported resistance genes including *tetA*, *tetB*, *tetC*, *tetD*, *tetE*, *tetG*, *tetK*, *tetL*, *tetM*, *tetO*, *tetS*, *tetAP*, *tetQ*, and *tetX* were

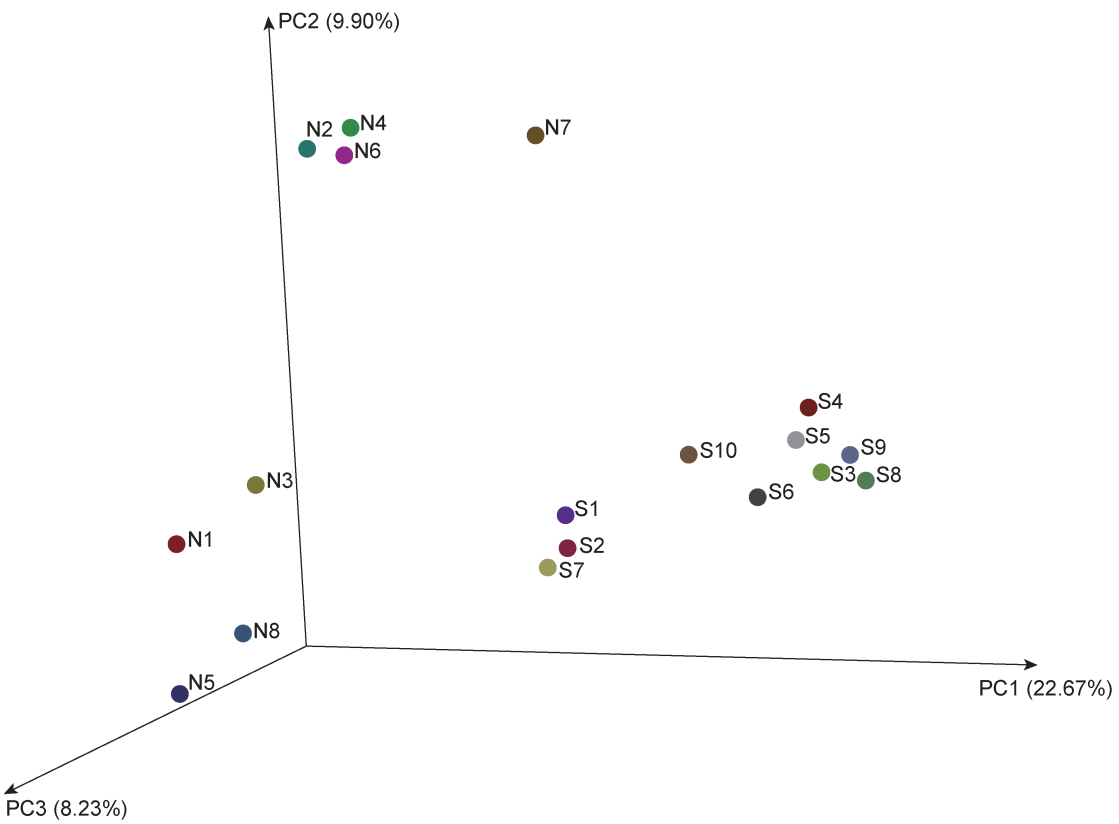
Tab. 1　The distribution of tetracycline resistant bacteria and tetracycline resistance genes in isolates from farmhouse and feedlot

Strains	Small farmhouse				Big feedlot			
	Total	*tetA*	*tetB*	*tetM*	Total	*tetA*	*tetB*	*tetM*
Acinetobacter johnsonii	1	0	0	1	0	0	0	0
Acinetobacter lwoffii	0	0	0	0	2	2	0	0
Brachybacterium alimentarium	0	0	0	0	1	0	0	1
Brachybacterium conglomeratum	0	0	0	0	1	0	0	0
Brachybacterium sp.	0	0	0	0	1	0	0	0
Corynebacterium sp.	0	0	0	0	2	1	0	0
Corynebacterium stationis	0	0	0	0	2	0	0	0
Enterococcus casseliflavus	0	0	0	0	3	0	0	3
Enterococcus faecalis	20	0	0	18	4	1	1	4
Enterococcus faecium	9	0	0	9	0	0	0	0
Enterococcus gallinarum	0	0	0	0	2	0	0	2
Escherichia coli	34	25	3	9	35	21	14	2
Escherichia fergusonii	2	0	0	0	1	1	0	0
Jeotgalicoccus halophilus	0	0	0	0	1	0	0	0
Pseudomonas putida	0	0	0	0	1	1	0	0
Psychrobacter pulmonis	0	0	0	0	2	2	0	0
Raoultella ornithinolytica	0	0	0	0	2	0	0	0
Shigella sp.	0	0	0	0	1	0	0	0
Sporosarcina aquimarina	1	0	0	0	0	0	0	0
Staphylococcus arlettae	1	0	0	0	0	0	0	0
Staphylococcus chromogenes	1	0	0	0	0	0	0	0
Staphylococcus equorum	0	0	0	0	2	1	0	2
Staphylococcus sciuri	0	0	0	0	1	1	0	1
Staphylococcus sp.	0	0	0	0	1	0	0	0
Total	69	25	3	37	65	31	15	15

tested in these antibiotic resistant isolates. Three of them including *tetA*, *tetB* and *tetM* were detected whereas others were not. As for the distribution analysis of resistance genes, *tetM* was mainly distributed in *Enterococcus* spp. isolates while *tetA* and *tetB* in *Escherichia coli* isolates. Two isolates of *Escherichia coli* isolated from the same chicken from farmhouse were found simultaneously containing *tetA*, *tetB* and *tetM*. Other resistant isolates such as *Escherichia fergusonii*, *Sporosarcina aquimarina*, *Staphylococcus arlettae*, *Staphylococcus chromogenes*, *Brachybacterium* sp., *Corynebacterium stationis*, *Jeotgalicoccus halophilus*, *Raoultella ornithinolytica*, *Staphylococcus* sp. did not harbor any detected tetracycline resistance genes even though they were cultured tetracycline resistant.

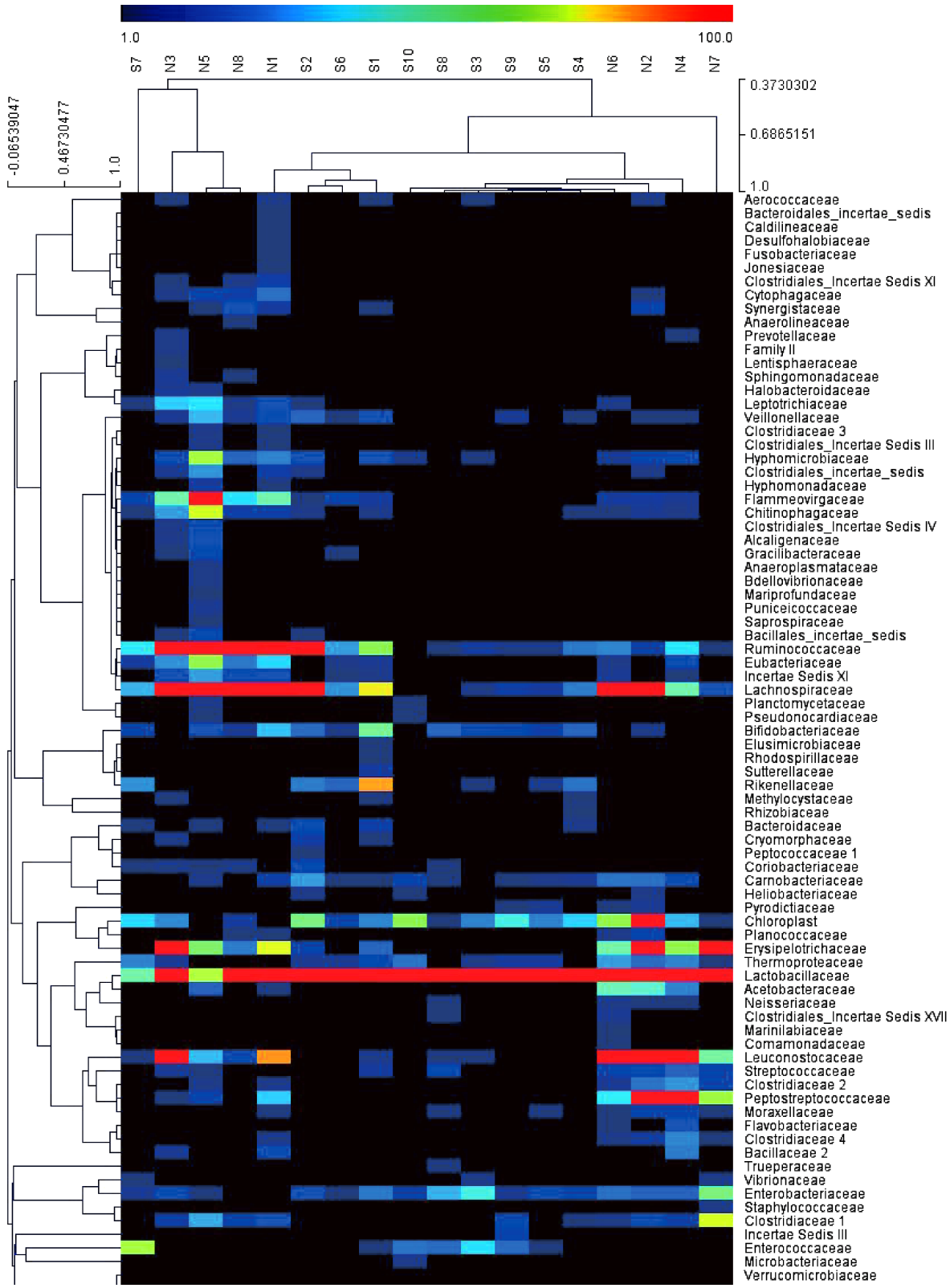
2.2 Sequence diversity

A total of 21 543 trimmed reads were retrieved with average sequence length of 200 bp. The mean Shannon diversity index in farmhouse group was 5.321 ± 0.590 and that in big feedlot group was 4.398 ± 0.440. The former was significantly higher than the latter (Mann-Whitney U test, $P = 0.008$). As shown in Fig. 1, the fecal samples of the same resource were inclined to cluster together. That suggested that fecal microbiota of the chickens from the same place may be more similar to each other than that from different places, even though there was a little difference among the chickens of the same resource. Furthermore, individual samples from farmhouse were much more dispersed than those from big feedlot.



Comparison of bacterial community composition reveals that the fecal microbiota from farmhouse-raised chickens was different from that from feedlot-raised chickens. Unweighted UniFrac was generated using Principal Coordinate Analysis (PCoA). The percentage of variation explained by each PCoA is indicated on the axes. The number beginning with "S" means the sample from feedlot. The number beginning with "N" means the chicken from farmhouse.

Fig. 1 Comparison of bacterial community composition



Analysis of abundance of bacterial lineages demonstrates that fecal microbiota cluster was based on their resources. The relative abundance of most common families (rows) was shown by the key to the top of the figure. The bacterial taxa present in the gut community of each chicken were summarized. Each sample was indicated along the *X*-axis, and the *Y*-axis indicates the relative abundance of each type (family) of bacteria present in that gut community. The bacterial taxa were listed at the right. Communities were clustered by hierarchical clustering using complete linkage of Euclidean distance matrix.

Fig. 2　Analysis of abundance of bacterial lineages

2.3 Family-level comparison of bacterial communities

Based on 16 rDNA V3 region pyrosequencing and analysis, the gene library revealed the presence of 151 bacterial families. Ninety-one families presented in the feces of chickens from feedlot and 120 families from farmhouse (data not shown). Fig.2 showed the distribution of the most common 77 families detected in feces. Among the detected families, Ruminococcaceae, Lachnospiraceae, Bifidobacteriaceae, Chloroplast, Lactobacillaceae, Enterobacteriaceae were abundantly distributed in all fecal samples. The families including Cytophagaceae, Leptotrichiaceae, Veillonellaceae, Hyphomicrobiaceae, Clostridiales_incertae_sedis, Erysipelotrichaceae, Leuconostocaceae, Streptococcaceae, Peptostreptococcaceae, and Clostridiaceae were much more commonly and abundantly distributed in chicken feces from farmhouse than those from feedlot. However, Enterococcaceae and Rikenellaceae were more abundant in chicken feces from feedlot than those from farmhouse.

3 DISCUSSION

It was reported that antibiotic use in agriculture can hasten the emergence of antibiotic resistance in bacteria[12]. Chicken is one of the major livestock which supply poultry meat we consume every day. The diversity and antimicrobial resistance profiles of bacteria in chicken gut can in some degree influence the rearing status of chickens. Further, the contamination of chicken feces in chicken meat, especially the pathogen contamination, is the major source for pathogen transmission to human food chain and can communicate with human microbiota[5-6]. Our study tried to reveal the differences in microbiota and antibiotic resistance distribution between chicken feces from big feedlot and small farmhouse. As shown in Tab.1, more than half of tetracycline resistant bacteria isolated from two sample groups were *Escherichia* and *Enterococcus*, suggesting that these species are the major antibiotic gene reservoirs in gut microbiota. Other species such as *Acinetobacter johnsonii*, *Acinetobacter lwoffii*, *Brachybacterium alimentarium*, *Raoultella ornithinolytica*, *Corynebacterium* sp., *Pseudomonas putida*, *Psychrobacter pulmonis*, *Staphylococcus equorum* and *Staphylococcus sciuri* were also detected to carry some resistance genes which suggested that resistance genes are distributed widely in the microbiota of chickens. Besides, it was shown that 19 tetracycline-resistance species were isolated from big feedlot, which were more diverse than those (8 species) from small farmhouse. It is known that sub-therapeutic doses of antibiotics for agricultural animals were used in chickens feeding in big feedlot[13]. Antibiotics can make bacteria evolve to antibiotic resistance[14-18]. It may be the reason that tetracycline resistant bacteria in chickens from big feedlot were more diverse than those from small farmhouse.

Tetracycline resistance genes were much more widely distributed in different species in the chickens from big feedlot than those from small farmhouse. A report shows that *tetB* was clustered with other antibiotic resistance genes on *Escherichia coli* chromosome[19]. In *Enterococci* and some other species, *tetM* has been found to be associated with conjugative transposons related to Tn916/Tn1545 family or some other mobile genetic elements[20-22]. The mobile elements make these antibiotic resistance genes much more easily transfer among species especially in the condition of antibiotics. Chicken feces are at risk to hasten the dissemination of antibiotic resistance genes if they were contaminated by antibiotic resistant bacteria. So we should evaluate the influences of antibiotic use (or the form of livestock rearing) in some big feedlots on the dissemination of

antibiotic resistance genes to human beings, even though they can provide much meat. Fortunately, some areas have forbidden the antibiotics as growth promoters in animal feeding[23].

The results of pyrosequencing of 16S rDNA V3 hyper-variable region showed that the microbiota in the chickens from small farmhouse was more diverse than that from big feedlot. Further analysis showed that the fecal samples of the same resource were inclined to cluster together. This suggested that fecal microbiota of the chickens from the same place may be more similar to each other than that from different places. Report shows that the gut of chicken harbors amounts of bacteria[6]. The biological factors such as disease state, treatment and so on, can bring a measurable difference in a gut bacterial community. In-feed administration of sub-therapeutic chlortetracycline alters the community composition and structure in the fecal flora of rat[24]. Sub-therapeutic antibiotic use in chickens from big feedlot may be accounted for the less diversity of microbiota in their guts. These indicated that the forms of livestock rearing (including diets, antibiotic use, chicken density and so on) may also affect the diversity of microbial composition in chickens from small farmhouse and (or) big feedlot. While the sample size of chickens enrolled in the study was small, the conclusion of the study should be further verified by large size samples in our future research.

Our study revealed that tetracycline resistant bacteria in the chickens from big feedlot were much more diverse than those from farmhouse by using culture-dependent method. Interestingly, the results of our culture-independent method show that the microbial composition in farmhouse chickens was more diverse than that in big feedlot. The results suggest that more attentions should be paid to the changes in the microbiota and antibiotic resistant bacteria in the chickens rearing in big feedlot, and the risks of dissemination of antibiotic resistance genes to human beings.

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