INFLUENCE OF LONG-TERM CHICKEN MANURE APPLICATION ON THE CONCENTRATION OF SOIL TETRACYCLINE ANTIBIOTICS AND RESISTANT BACTERIA VARIATIONS

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Abstract. A fixed field experiment was carried out to investigate the influence of different amounts of cured and fresh chicken manure (low chicken manure: 300 kg·667 m^{-2}, high chicken manure: 600 kg·667 m^{-2}) on the concentration of tetracyclines (TCs); number of microbes, including antibiotic resistant bacteria; organic matter content; and enzyme activity in soil. Results show that after a one-year application of chicken manure, the quantity of soil bacteria was 3.2-4.3 times that of the control soil. The quantity of soil bacteria and actinomycetes in cured chicken manure (CCM) treatments was significantly higher than in fresh chicken manure (FCM) treatments by 17.0%-33.9% and 201.2%-271.2%, respectively. The application of low cured chicken manure (L-CCM) significantly reduced the number of fungus, which was 83.8% lower than the control. The application of CCM significantly increased the activities of soil urease and catalase by 81.9%-103.0% and 7.9%-17.9%, respectively, compared to the control. After applying chicken manure in soil where *Ipomoea aquatic* Forsk was planted, the soil antibiotic residues increased by 3.6%-27.5%. When applying chicken manure to soil where *Lactuca sativa* L. or *Brassica juncea var. gemmifera* was planted, the soil antibiotic residues decreased by 19.4%-52.2% and 22.6%-26.6%, respectively. After one year, the antibiotic residues in soil treated with FCM and CCM were higher than the control level by 176.5%-217.9% and 168.5%-191.5%, respectively. The number of resistant bacteria in CCM treatments was significantly higher than that in FCM treatments and in the control. The number of resistant bacteria in the L-CCM treatment was the highest, wherein the quantity of resistant bacteria to oxytetracycline, tetracycline and chlortetracycline was higher than that in other treatments by 11.6%-339.6%, 127.0%-635.4% and 32.2%-130.9%, respectively. The drowned planting mode may worsen soil tetracycline antibiotic residues, when compared to drought planting. Long-term application of FCM may cause residue and accumulation of tetracycline antibiotics in soil; hence the hazard to the environment is worthy of further study.
Keywords: *chicken manure, tetracycline antibiotics, resistant bacteria, soil enzyme activity, planting mode*

Introduction

In recent years, the adsorption, migration, and conversion of novel organic pollutant antibiotics in soil-water-plant have become an area of intense research. Antibiotics are widely used to reduce disease in the livestock and poultry industries. In 2010, the annual administration of antibiotics in the livestock and poultry industry reached approximately 63,151 tons, and it is expected to increase by 67% by 2030 (Boeckel et al., 2015). China accounts for 60%-65% of the world’s total antibiotic use (Tasho and Cho, 2016). Furthermore, production of livestock and poultry manure from large-scale farms in China can reach 2.1 billion tons, 80% of which are directly applied to farmland without comprehensive treatment (Sun et al., 2017). In eastern China, the concentration of antibiotic residues in livestock and poultry manure can be as high as 1420 mg·kg^{-1}

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(Chen et al., 2012). Tetracycline antibiotics are one of most widely used classes of antibiotics in the world, and include tetracycline (TC), oxytetracycline (OTC) and chlortetracycline (CTC). Liguoro et al. (2003) detected OTC in livestock and poultry manure at concentrations up to 871.7 mg·kg^{-1} and 115.5 mg·kg^{-1}, respectively, and CTC in a pig manure treatment pond was detected at concentrations up to 1.0 mg·kg^{-1}. Hamscher et al. (2002) detected TC and CTC in livestock manure at 4.0 mg·kg^{-1} and 0.1 mg·kg^{-1}, respectively.

Farmland is a main site where antibiotics are spread. Livestock and poultry manure are applied to farmlands as organic fertilizer, which is one of the main mechanisms in which antibiotics enter the soil (Kemper, 2008). Tetracycline antibiotics are recalcitrant and easily accumulate in soils, thereby posing potential threats to the environment and human health (Pan and Chu, 2017). Hamscher et al. (2002) studied the concentration of antibiotic residues in soils that received long-term application of livestock and poultry manure. They found that the average TC concentration was 86.2 μg·kg^{-1} in 0-10 cm soil, 198.7 μg·kg^{-1} in 10-20 cm soil, and 171.7 μg·kg^{-1} in 20-30 cm soil. After application of livestock and poultry manure, the highest concentrations of OTC, TC and CTC in surface soil (0-30 cm) were 27 μg·kg^{-1}, 443 μg·kg^{-1}, 93 μg·kg^{-1}, respectively. Of the 14 sampling points, TC concentrations exceeded the emission reference value (100 μg·kg^{-1}) prescribed by the European Medicine Agency at 3 points (Sarmah et al., 2006). A study investigating the soil antibiotic content in northern Zhejiang, China, found that the average residual OTC, TC and CTC in manure-treated topsoil was 38-fold, 13-fold and 12-fold more or higher than without application of livestock and poultry manure in farmland (Zhang et al., 2008).

After entering into the environment, manure-associated antibiotics are highly recalcitrant. Moreover, even extremely low concentrations of antibiotics can significantly affect microbial activity (Tasho and Cho, 2016). Yang et al. (2010) found that the growth of most predominant bacteria in the wheat root system was fully suppressed in soils with OTC and TC concentrations of 0.05 mg·L^{-1} and 0.5 mg·L^{-1}, respectively. Many antibiotic resistant bacteria have been detected and separated from soil (Brandt et al., 2009). The antibiotic resistant bacteria, which are generated by residual antibiotics in livestock manure, may enter plant systems via the root, and then enter into the human body via the food chain; thus leading to drug resistance in humans (Zhang et al., 2012). This will bring serious threats to human health. If antibiotic-resistance continues to grow, numerous human and animal diseases will be untreatable (Hu et al., 2015; Tasho and Cho, 2016). It is of great importance to investigate the influence of different fertilization methods and manure types on the environmental deposition of antibiotics (Pruden et al., 2013). Furthermore, drought and drowned cultivation may effect on the decomposition of antibiotics (Hu et al., 2015). Therefore, a fixed field experiment was carried out to analyze tetracycline residues and the variation in antibiotic resistant bacteria after one-year of application using different types and amounts of chicken manure. Furthermore, the influence of different planting modes (drought planting mode and drowned planting mode) on tetracycline residues was investigated.

Materials and method

Three vegetables (*Ipomoea aquatic* Forsk (drowned cultivation mode), *Lactuca sativa* L. and *Brassica juncea* var. *gemmifera* Lee et Lin (drought cultivation mode))

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were used in the present study. A fixed field experiment was conducted from March, 2015 to March, 2016 in the Shengde Village, Sansheng Town, Beibei District of Chongqing (29°56′16″N，106°38′16″E). The contents of organic matter, total nitrogen, alkali-degradable nitrogen, available K, available P and the soil pH were 53.4 $g \cdot kg^{-1}$, 1.04 $g \cdot kg^{-1}$, 120.8 $mg \cdot kg^{-1}$, 90.5 $mg \cdot kg^{-1}$, 75.0 $mg \cdot kg^{-1}$, and 4.52, respectively. The average concentrations of quartz, montmorillonite, kaolinite and illite accounted for 14.12%, 16.82%, 7.14% and 61.91% of the total soil clay minerals in the 0-20 cm depth, respectively. Chicken manure was collected from a large chicken farm near the fixed field experimental site. The content of TC, OTC and CTC in fresh chicken manure (FCM) was 1021.64, 272.03 and 13937.18 $\mu g \cdot kg^{-1}$, respectively; the content of TC, OTC and CTC in cured chicken manure (CCM) was 18.37, 29.96 and 69.03 $\mu g \cdot kg^{-1}$, respectively.

Five plot experiments were conducted without chicken manure (CK), low-fresh chicken manure (L-FCM; 300 $kg \cdot 667m^{-2}$), high-fresh chicken manure (H-FCM; 600 $kg \cdot 667m^{-2}$), low-cured chicken manure (L-CCM; 300 $kg \cdot 667m^{-2}$), and high-cured chicken manure (H-CCM; 600 $kg \cdot 667m^{-2}$). The area of each square test plot was 6 m^2. The experiment was performed in triplicate, and arranged randomly. Before planting, chicken manure was applied according to the predetermined amount. Farmland irrigation and pest control in the experimental field were carried out according to the regular practices of peasant households. Before applying the chicken manure and when harvesting the vegetables, topsoil samples (0-15 cm) were randomly collected from different points using a soil auger sterilized with 70% ethanol. Soil samples were put in an icebox and then taken back to the laboratory. A portion of the soil samples were stored at 4°C. Refrigerated samples were used to test the antibiotic content, soil microorganism abundance, antibiotic resistant bacteria abundance and soil enzyme activity. The remaining portion was air-dried and then used to test the organic content.

Determination of organic matter and enzyme activity in soil

The organic content in the soil was measured as described by Nelson and Sommers (1982). Soil catalase activity was measured by the permanganate titration method and expressed as the 0.1 N $KMnO_4$ amount (mL) per gram of soil and 20 min (He et al., 2015). Urease activity was measured by indophenol blue colorimetry and expressed as the NH_3-N amount (μg) per gram of soil (He et al., 2015).

Quantification of microorganisms and antibiotic resistant bacteria in soil

Bacteria were cultured in beef extract water peptone agar medium. Fungi were cultured in Martin-Bengal red medium. Actinomycetes were cultured in improved Gause No.1 medium. All microbes were quantified as per the colony forming unit method (Negreanu et al., 2012). The quantity of antibiotic resistant bacteria was measured as described by Negreanu et al. (2012).

Analysis of tetracycline antibiotics in soil and manure

Tetracyclines in the soil and in livestock and poultry manure were extracted and measured as described by Jacobsen et al. (2004). Measurements were conducted using a high performance liquid chromatograph (HPLC, Shimadzu, Japan) equipped with SPD-20A UV-detector. The chromatographic column (Inertsil ODS-3; 4.6×150 mm, 5 μm) was run at a mobile phase flow rate of 1 $mL \cdot min^{-1}$, injection volume of 10 μL, UV

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detection wavelength of 355 nm, and a sampling time of 25 min. The average recovery rates of OTC (58.3%), TC (57.1%) and CTC (87.4%) were measured by the addition standard method. All variable coefficients were under 11.04%.

Statistical analysis

The data were subjected to three-way univariate ANOVA using SPSS version 21.0 (IBM Corp., Armonk, NY, USA). Parameters for soil organic content, enzyme activity, soil resistant bacteria and soil-associated tetracycline residues were analyzed. Additionally, the correlation between the application amount of chicken manure and tetracycline residues was determined. Prior to ANOVA, normal probability and residual plots were constructed for each dataset and examined for unequal variance and deviations from normality among the residuals. All data fulfilled the conditions for equal variance and normality. Means were considered statistically significant at $P \leq 0.05$ using Fisher's least significant difference test.

Results and analysis

Soil organic content and enzyme activity

After application of chicken manure, the content of organic matter in the soil increased by 1.0%-3.2% when compared with the control (*Table 1*). No significant difference in soil organic matter content was found among treatments. Soil catalase activity in FCM and CCM treatments significantly increased by 84.3%-91.5% and 81.9%-102.9%, respectively, compared to the control (*Table 1*). Soil catalase activity in the H-CCM treatment (600 kg·667m^{-2}) was 11.7% higher than that in the L-CCM treatment (300 kg·667m^{-2}). Compared with the control, soil urease activity in the H-FCM treatment (600 kg·667m^{-2}) decreased by 34.2%. Soil urease activity in the H-CCM treatment was 17.9%, 18.3% and 79.1% higher than the control treatment, L-FCM treatment (300 kg·667m^{-2}) and H-FCM treatment, respectively.

Table1. *Effects of different chicken manure on the contents of organic matter, enzyme activities and microbial quantity in soil after one year*

Treatment	Organic matter/g·kg^{-1}	Catalase activity/ml·h^{-1}g^{-1}	Soil urease activity/μg·g^{-1}	Bacteria×10^{6} /CFU·g^{-1}	Actinomycetes×10^{5} /CFU·g^{-1}	Fungi×10^{4} /CFU·g^{-1}
CK	54.39±3.84a	0.57±0.06c	0.26±0.04a	7.22±0.10c	5.87±0.26b	23.44±3.70a
L-FCM	55.20±4.39a	1.08±0.06ab	0.26±0.06a	23.02±2.56b	2.81±0.26c	10.23±2.56b
H-FCM	56.11±4.59a	1.04±0.02ab	0.17±0.02b	23.13±4.41b	2.23±0.60c	6.87±1.49bc
L-CCM	54.94±4.05a	1.02±0.03b	0.28±0.03a	30.81±2.57a	8.47±1.56a	3.80±1.29c
H-CCM	55.34±4.37a	1.11±0.01a	0.30±0.05a	27.06±1.51ab	8.29±0.55a	22.69±3.02a

Different letters (a, b, c) indicate significant difference at $P \leq 0.05$ among different treatments

The abundance of soil microorganisms was as follows: bacteria > actinomycetes > fungi (*Table 1*). Application of chicken manure significantly increased the quantity of bacteria, which was 3.2-4.3 times that in the control. The bacterial abundance in the L-CCM treatment was significantly larger than that in L-FCM treatment, with increases of 17.0%-33.9%. The number of actinomycetes in the CCM treatments significantly increased by 41.3%-44.4%, compared with the control.

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However, the abundance of actinomycetes in the FCM treatments was lower than in the control, with reductions of 52.1%-61.9%. The actinomycetes abundance in CCM treatments with identical application amounts was 201.2%-271.2% higher than in FCM treatments. The application of chicken manure reduced the abundance of fungi in the soil with L-CCM treatments by 62.9%, compared with that in L-FCM treatments.

Quantity of antibiotic resistant bacteria in the soil

The abundance of soil antibiotic resistant bacteria in the same treatments was as follows: Anti-OTC > Anti-TC > Anti-CTC (*Fig. 1a*). The quantity of resistant bacteria treated with chicken manure was higher than that in control soil, with the CCM treatments being significantly higher than the FCM treatments. The highest abundance of resistant bacteria was found in the L-CCM treatment, wherein the number of Anti-OTC, Anti-TC and Anti-CTC in the soil was 11.6%-339.6%, 127.0%-635.4% and 32.2%-130.9% higher, respectively, than in the other treatments.

Application of chicken manure increased the proportion of resistant bacteria in the soil (*Fig. 1b*). The proportions of Anti-TC treated with chicken manure were significantly higher than the control by 42.9%-102.2%.

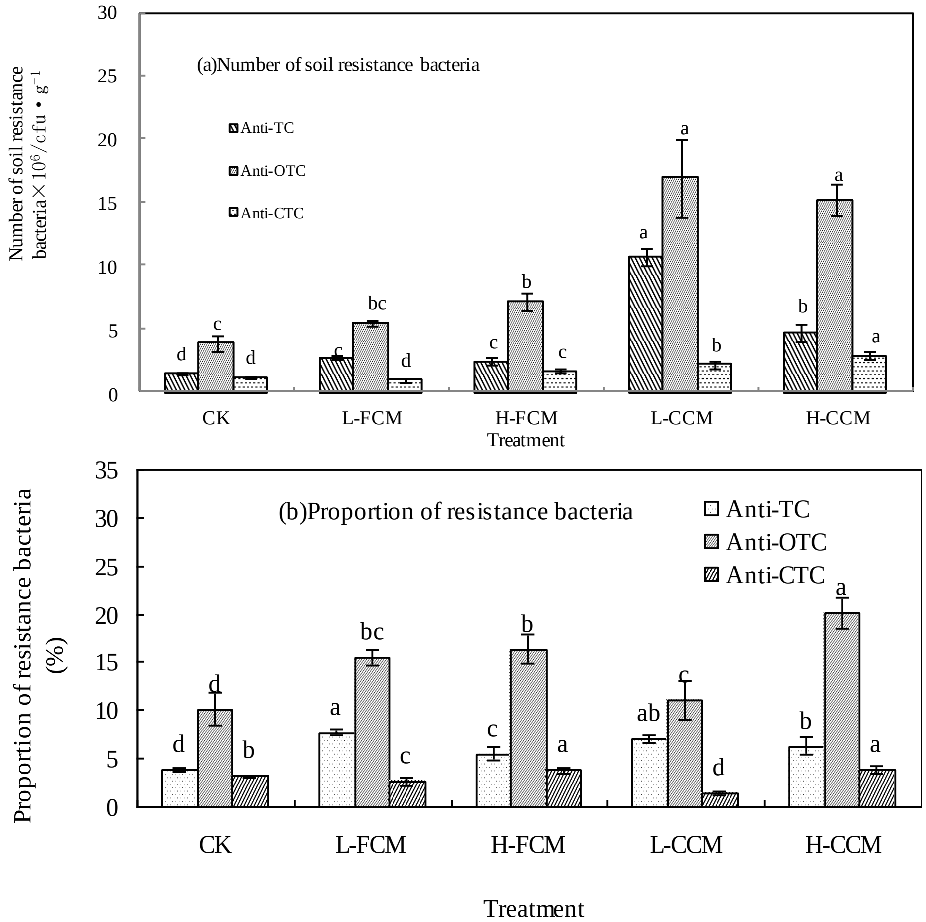

***Figure 1.** Effects of different chicken manure treatments on the amount and proportion of soil tetracycline resistant bacteria. Anti-TC: Tetracycline resistant bacteria; Anti-OTC: Oxytetracycline resistant bacteria; Anti-CTC: Chlortetracycline resistant bacteria; Different letters (a, b, c) indicate significant difference at $P \leq 0.05$ among different treatments*

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The proportions of Anti-OTC in FCM treatments and H-CCM treatments were also significantly higher than that in the control. The proportions of Anti-CTC in H-FCM treatments and CCM treatments were higher than that in the control, while the proportion of Anti-CTC in the L-CCM treatments was lower than that in control by 54.8%.

Variation of tetracycline antibiotic residues in soil

The total amount of soil tetracycline after harvesting *Ipomoea aquatica* Forsk in the L-FCM, H-FCM, L-CCM and H-CCM treatments increased by 7.3%, 27.5%, 19.4% and 3.6%, respectively, compared with that before planting (*Fig. 2a*).

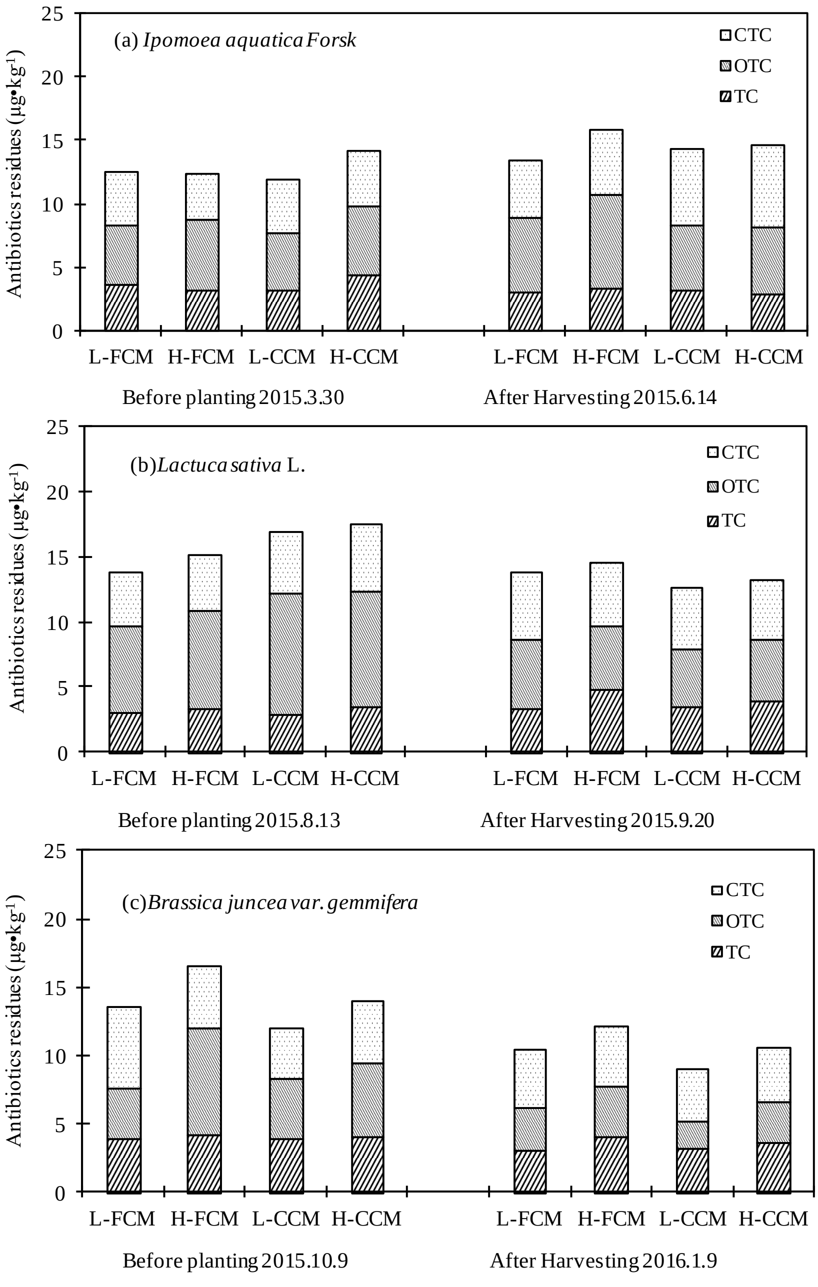

Figure 2. *The concentration changes of tetracycline antibiotics in soil during the application of chicken manure in different vegetable crop cropping patterns*

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The highest amount of soil tetracycline was found in the H-FCM treatment. The OTC amount the in L-FCM and H-FCM treatments increased by 25.2% and 32.8%, respectively. The amount of CTC in soils treated with chicken manure generally increased, with the highest CTC content observed for the H-CCM treatment, which increased by 52.2% compared with that before planting *Ipomoea aquatica* Forsk.

The total amount of soil tetracycline decreased after harvesting *Lactuca sativa* L. compared with before planting (*Fig. 2b*). The total amount of antibiotics after harvesting *Lactuca sativa* L. in the L-CCM and H-CCM treatments decreased by 33.6% and 32.7%, respectively. Furthermore, the OTC content in H-CCM and L-CCM treatments decreased by 47.3% and 52.2%, respectively, compared with that before planting *Lactuca sativa* L.

The total amount of soil tetracycline in the L-FCM, H-FCM, L-CCM and H-CCM treatments after harvesting *Brassica juncea var. gemmifera* decreased by 22.6%, 26.6%, 24.9% and 24.2%, respectively, compared with before planting. Moreover, the soil OTC contents decreased by 14.7%, 52.1%, 55.5% and 45.1%, (*Fig. 2c*).

The total amounts of soil tetracycline after one year of treatment with L-FCM, H-FCM, L-CCM and H-CCM increased by 176.5%, 217.9%, 168.5% and 191.5%, respectively, compared to the control (*Fig. 3*). The highest total amount of soil tetracycline (13.069 $\mu g\cdot kg^{-1}$) was found in the H-FCM treatment. Soil tetracycline residues in high chicken manure treatments (600 $kg\cdot 667m^{-2}$) were higher than that in the low chicken manure treatments (300 $kg\cdot 667m^{-2}$) by 8.5%-15.0%, and approximately twice that of the control. The antibiotic residues in the FCM treatments were higher than that in the CCM treatments by 3.0%~9.1%. In general, the TC residues in the soils treated with L-CCM was the lowest, while that with H-FCM was the highest.

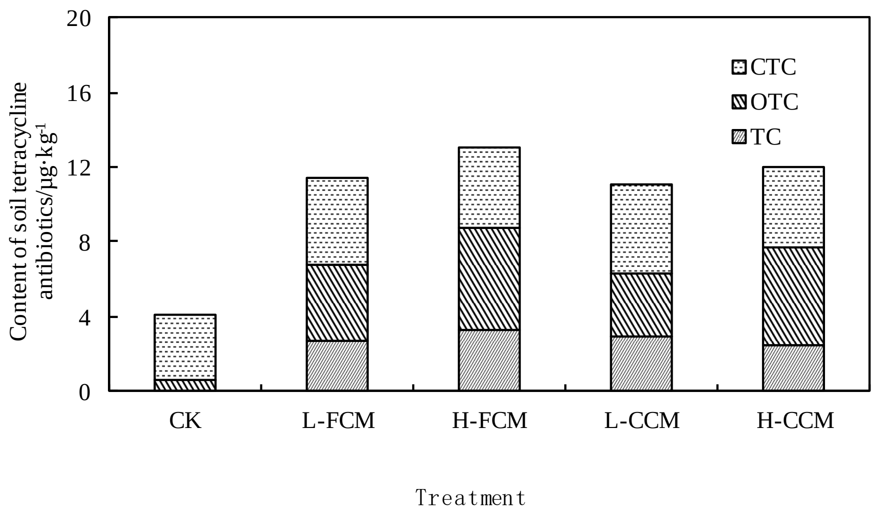

Figure 3. *The amount of soil tetracycline antibiotics in the chicken manure treatments after one year*

The application amount of chicken manure significantly and positively correlates with the total amount of soil tetracycline (r = 0.880), wherein the TC and OTC residues are positively correlated with the application amount of chicken manure ($r = 0.648$, $P < 0.05$; $r = 0.936$, $P < 0.01$). Conversely, the CTC residue does not correlate with the chicken manure application amount (*Table 2*).

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Table 2. *The correlations of chicken manure application amount and soil tetracycline antibiotic residues*

	Application amount of chicken manure	**Soil TC**	**Soil OTC**	**Soil CTC**	**ΣTCs**
Application amount of chicken manure	1				
Soil TC	0.648*	1			
Soil OTC	0.936**	0.714*	1		
Soil CTC	0.323	0.716*	0.458	1	
ΣTCs	0.880**	0.874**	0.959**	0.645*	1

* or * * indicates significant difference of the correlations of chicken manure application amount and soil tetracycline antibiotic residues at $P \leq 0.05$ or $P \leq 0.01$ level. ΣTCs indicates the total amount of the three tetracycline antibiotics

Discussion

With the application of organic manure fertilizers, antibiotics enter and then accumulate in soil. Antibiotic residues in soil are largely determined by the soil characteristics, such as pH, organic matter content and mineral composition. Soil mineral and organic matter fractions could be the primary antibiotic adsorption sites (Qiu et al., 2004). In the present study, no significant difference in soil organic contents was found between different treatments after one year of applying different chicken manures. This may be because the difference in chicken manure application amounts among treatments was not significant or because the test period was only one year; therefore, further long-term in-situ monitoring may be required.

Soil microorganisms and enzymes are important components of soil biochemical characteristics, which play important roles in nutrient transformation and organic matter decomposition (Zhang et al., 2012). Soil enzymes are important components of the soil ecosystem and a key index for evaluating ecological environment and fertility of the soil (Yao et al., 2010). In the present study, the application of chicken manure significantly increased catalase activity in the soil; however, no significant difference in soil catalase activity was found between the FCM and CCM treatments. This result is similar to that of Yao et al. (2010). In this study, soil urease activity after application of FCM was lower than after application of CCM. The reasons may be because FCM normally results in strong acidity (pH 3.6-4.7), which is far from the suitable pH value of urease (6.5-7.0) (Dodd et al., 1987). In addition, FCM is generally in an organic or slowly-released state that cannot be absorbed and used by plants, leading to slow fertilizer efficiency, which affects urease activity (Liu et al., 2003). Application of CCM increases soil urease activity, which is consistent with results published by Jin et al. (2013). In the present study, the quantity of soil bacteria in the CCM treatments was larger than that in FCM treatments. This may be caused by the strong acidity and slowly-released nutrients from FCM, which affects the urease activity, thus resulting in a decreased bacterial abundance.

The quantity of soil bacteria in the high chicken manure treatments (600 $kg \cdot 667m^{-2}$) was generally lower than that in the low chicken manure treatments (300 $kg \cdot 667m^{-2}$). This indicates that the application amount of organic fertilizer affects the quantity of soil bacteria. The application of CCM caused a significant increase in the quantity of actinomycetes, while FCM limited the quantity of actinomycetes. The number of soil

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fungi decreased after the application of chicken manure. This result further confirms that appropriate application of CCM is beneficial for the growth of bacteria and actinomycetes in soil, and it also limits the growth of soil fungi and reduces harm to plants. These results are in agreement with those published by Yang et al. (2007).

The application of chicken manure increased the number of resistant bacteria, wherein the CCM treatment increased this subpopulation more than FCM did. This may be because the antibiotic degradation products in CCM have a larger influence on soil resistant bacteria. In the present study, compared to the control, appropriate application of CCM reduced the proportion of Anti-OTC, which is consistent with the research results of Zhang et al. (2012). The quantity and proportions of Anti-CTC in the soil after application of different chicken manures, and the proportion of soil Anti-CTC in high chicken manure treatments, was larger than that in low chicken manure treatments. This is probably because the high application amount of chicken manure (600 $kg{\cdot}667m^{-2}$) improves the soil physicochemical properties and changes its adsorption capacity for CTC (Pils and Laird, 2007). In the present study, the application of CCM greatly reduced the number and proportion of resistant bacteria, while the application of FCM increased these values, which is similar to the results reported by Yang et al. (2014).

During the yearlong application of chicken manure, three crops (*Ipomoea aquatica* Forsk, *Lactuca sativa* L. and *Brassica juncea var. gemmifera*.) were planted using two different cultivation modes (drowned and drought cultivation). The soil antibiotic residues after harvesting *Ipomoea aquatica* Forsk in drowned cultivation mode increased, while the residues after harvesting *Lactuca sativa* L. and *Brassica juncea var. gemmifera* in drought planting mode decreased. This may be because, in drought cultivation using *Lactuca sativa* L. and *Brassica juncea var. gemmifera,* the water downwardly infiltrated and evaporated, then the antibiotics also downwardly infiltrated under the plough layer, or the soil physicochemical property changed due to lack of water; therefore the antibiotic residues in plough layer soil decreased (Du and Liu, 2011; Hu et al., 2015). After application of chicken manure for one year, the soil antibiotic residues significantly increased, wherein the residue in the FCM-treated soil was higher than that in the CCM-treated soil. The reasons may be because the FCM is acidic. With the increase of soil pH and ion strength, the absorption capacity of the soil for tetracycline decreased (Gündoğan et al., 2004). The antibiotic residues in high chicken manure treatments (600 $kg{\cdot}667m^{-2}$) were higher than that in low chicken manure treatments (300 $kg{\cdot}667m^{-2}$), which is similar to the results reported by Zhang et al. (2015). The total antibiotic content in the soil was significantly positively correlated with the application amount of chicken manure. This result further confirms that the higher the amount of manure application are, the higher the soil antibiotic residues. However, the soil CTC content was not correlated with the application amount of manure, possibly because the soil CTC content is correlated with the type of manure or soil background value; however, these results should be validated by further long-term studies.

Conclusion

After one year, soil catalase activity increased in chicken manure-treated soils. Soil urease activity increased in CCM treatments. The quantity of bacteria and actinomycetes significantly increased in L-CCM treatments, while the number of fungi significantly decreased. The application of CCM significantly increased the quantity of actinomycetes, while the application of L-CCM significantly reduced the proportion of

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antibiotic resistant bacteria. The antibiotic residues in CCM-treated soil were lower than that in FCM-treated soil. The antibiotic residues in high chicken manure treatments (600 kg·667m^{-2}) were higher than that in low chicken manure treatments (300 kg·667m^{-2}). The cultivation mode also affected antibiotic residues in soil, wherein the drowned cultivation mode may worsen the tetracycline antibiotics residues in the soil. Long-term application of FCM may cause residue and accumulation of tetracycline antibiotics in soil and harm to the ecological environment.

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