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Short-term thermophilic treatment cannot remove tetracycline resistance genes in pig manures but exhibits controlling effects on their accumulation and spread in soil

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HIGHLIGHTS

- Short-term thermophilic treatment cannot remove TRGs in pig manures.
- Treated pig manures exhibits controlling effects on accumulation of TRGs in soil.
- Decline of gram-negative TRB contributes to the controlling effects.

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ABSTRACT

In this work, a microcosm experiment was conducted to merely mimic thermophilic phase in aerobic composting with pig manures in order to explore: (i) the effect of thermophilic phase in composting on the abundances of tetracycline resistance genes (TRGs); and (ii) the impacts of the treated manures on the abundances of TRGs in soil. It was found that 4 days of thermophilic process reduced the abundance of TRGs in pig manures by ~1 lg unit compared to the samples without treatments, suggesting that other phases in composting may play significant roles in removal of TRGs. Once pig manures with thermophilic treatment were applied to soil, TRGs abundances decreased to the levels in unfertilized soil. With correlation analyses, it was concluded that pig manure derived tetracycline-resistant bacteria (TRB) and nutrients exerted different effects on TRGs abundances in soil. In conclusion, short-term thermophilic treatment cannot remove tetracycline resistance genes in pig manures but exhibits controlling effects on their accumulation and spread in soil. Nutrients enrichment in soil following manuring of treated pig manures, together with a large proportion of gram-positive TRB left in treated pig manures with less risk to TRGs spread, contributed to the controlling effects.

1. Introduction

As a class of broad-spectrum agents, tetracyclines (TCs) have been used against a wide range of pathogenic bacteria in both humans and animals for long time [1]. TCs are used mainly for treatment and prophylaxis in the livestock than in human [2,3], which results in the selection of resistant animal pathogens through a horizontal gene transfer (HGT) by means of mobile genetic elements [4–6]. It was reported that the average antibiotic consumption per person in China is nearly 10 times more and far more consumption per pig than those in the United States [7,8]. Consequently, animals (including humans) manures possess the highest number of antibiotic resistance genes (ARGs), especially tetracycline resistance genes (TRGs) [9]. In rural China, pig manures are often applied as organic fertilizer directly to the soil without any treatment. Being a major source of antibiotic pollution [8], they lead to the large-scale pollution of soil, water, and harm to humans via food chain [10–13]. Thus, how to biosafety disposal of pig manures before field application is a big concern in China.

Researchers are devoting to develop methods for inactivation of ARGs in the environment. Recently, a good review article summarized some methods involved these years [14], and the following technologies are being considered, such as photocatalytical processes [15], sorption [16], plant uptake and phytodegradation [17], biodegradation [18], and nanotechnology [19], etc. Apart from these, composting of pig manures is supposed to be a potential method for manure management, for both antibiotics and abun-

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dances of some ARGs especially TRGs generally decline during the process [20–23]. Most of the declines during aerobic composting were attributed to thermophilic periods, during which many gram-negative bacteria with TRGs were killed or inactivated [20,24,25]. However, if thermophilic phase during aerobic composting is the key or the main factor for decrease of TRGs is not fully known, because there may be some other diverse and uncontrollable conditions that could affect the process. During the actual composting process, for example, shifts of C/N ratios, humidity, etc. could affect the bacterial communities and thus affect the abundances of TRGs [26,27]. In fact, there are opposite results obtained by some researchers. Some found out that ~50 days of composting could remove TRGs completely [20,28], but Wang et al. pointed out that thermophilic composting of swine manure could effectively remove antibiotics but not ARGs [29]. Now that thermophilic process is vital for removing ARGs from manures during composting [30], it is necessary to uncover the effects of the process on the removal of TRGs from manures. This will help to assess and improve the composting technology. Besides, a report has shown that the total abundance of TRGs in soil was not significantly reduced by compost manuring [31]. However, apart from thermophilic phase, if other phases in composting would induce increases in TRGs, it may result in a failure to control TRGs in soil. Therefore, it is also important to realize the impacts of pig manures merely with thermophilic treatment on TRGs abundances in soil.

In this present study, a microcosm experiment was conducted to merely mimic the thermophilic phase in aerobic composting in order to explore: (i) the effect of thermophilic phase in aerobic composting on the abundances of TRGs during the processing time; and (ii) the impacts of composted manures merely through thermophilic process on the abundances of TRGs in soil.

2. Materials and methods

2.1. Pig manure

Pig manures were collected from a pig farm with an eleven year feeding history in Qinfeng Town, Yangzhou City, where yielded about 1000 pigs every year. With HPLC–MS/MS method [32–34], tetracycline contents in manure samples were $986.3 \pm 39.4\ \mu g\ kg^{-1}$. Briefly, tetracycline was extracted with 25 mL of ethylenediaminetetraacetic acid (EDTA) solution (pH 12) from 5 g of manure sample, followed by an ultrasonic agitation for 30 min before centrifugation. After filtration with 0.22 μm filter, the concentrated extract was separated using an Endeaorsil C18 column in a Thermo Finnigan Surveyor HPLC system (Thermo Scientific, MA, USA). The mobile phase included a mixture of methanol and water plus 0.1% formic acid at a ratio of 40:60 (v/v) with a flow rate of $0.25\ mL\ min^{-1}$. The detection was performed using a TSQ Quantum Ultra AM (Thermo Scientific) equipped with an APCI ion source (IonMax) and operated in the positive ion mode. Besides normal feeds, fattening pan age (Loushi Co. Ltd.) with $21.57 \pm 16.33\ \mu g\ kg^{-1}$ of TCs was used as growth promoters at the rate of 2.5 kg per pig daily. Fresh pig manures were collected and taken to the laboratory for immediate use.

2.2. Mimic of thermophilic phase in aerobic composting

A total of twenty-five sterile Petri dishes (150 mm × 33 mm), each containing 50-g of pig manures, were prepared first. Moisture contents of each manure samples were adjusted to 55% using sterile ddH_2O [35,36]. The moisture contents were calculated according to the formula: water weight (g)/dry soil weight (g) × 100%, where the dry soil weight was determined after drying to constant weight in an oven at 110 °C for about 8 h [37]. Each Petri dish was placed in an incubator at 60 °C, a common thermophilic temperature for aerobic composting [38,39], with 60% humidity for 96 h. Of 25 Petri dishes, every five dishes (namely five replicates) were randomly taken out after 0, 12, 24, 48 and 96 h of incubation for determinations of absolute and relative abundances of TRGs. At each of the above-mentioned sampling timepoint, small quantities of samples were received a routinely serial dilution, and 100 μL of bacteria suspension were plated onto LB agar plates containing 0 or 30 μg/ml of TC, separately. After 48 h of incubation at 28 °C without light, 50 bacterial clones were randomly picked from appropriate plates with the same dilution, followed by an identification of spore-forming bacteria by endospore staining and microscopic examination. In view of the heat-resistant characteristics of spore-forming bacteria, the proportions of which to the total cultivable bacteria were calculated. This could help to discuss which groups of bacteria have possible relevance to the effects of high temperature-processed pig manures on TRGs abundances in soil.

2.3. Effects of high temperature-processed pig manures on TRGs abundances in soil

About 2.5 kg of soils were collected from deep layers (>60 cm) at five locations (each about 0.5 kg) in Yangzhou University. Soils were then mixed together. The characteristics of the soil samples were as follows: pH of 6.27 with a soil-water ratio of 1:1, organic matter of 11.07 g/kg, and cation exchange capacity of 9.34 cmol/kg. After pulverization and sieving (2 mm), soils were mixed evenly with pig manures with different treatments mentioned above in Petri dishes ($\varphi = 15$ cm) at a rate of 0.4% according to the traditional fertilization recommendation. Each dishes contained 100 g of soil. Fertilized soils were placed in an incubator at 25 °C and incubated for 0, 14 and 28 days. Soils without fertilization were also set up as blank controls. Each treatment was replicated five times. On day 0, 7, 14 and 28, soils were sampled for further determination of TRGs abundances.

2.4. Real-time quantitative PCR (q-PCR) assays for TRGs abundances

Total microbial DNA was extracted from manure and soil samples with Power-Soil™ DNA Isolation Kit (MO BIO Laboratories Inc., CA, USA) according to the manufacturer's instructions. Six TRGs, namely *tet*B, *tet*C, *tet*M, *tet*O, *tet*T, and *tet*Z genes (detectable genes in the soil by using the PCR approach in advance), were amplified with the primers referred to our previous work [33]. The PCR was performed using Bio-Rad Miniopticon (Bio-Rad Laboratories, CA, USA) with SYBR Green I detection for estimating the copy numbers of TRGs. A total of 20 μL reaction system contained 10 μL of iTaq Universal SYBR Green Supermix, 0.4 mM of each primer, and 10 ng of template DNA. The reaction procedure consisted of: 95 °C for 1 min, followed by 40 cycles of 94 °C for 10 s, 61 °C, 68 °C, 55 °C, 60 °C, 46 °C, and 61 °C for 45 s (corresponding to *tet*B, *tet*C, *tet*M, *tet*O, *tet*T, and *tet*Z genes, respectively), and the subsequent disassociation curve analysis. For normalization purposes, the copies of 16S rRNA genes were also quantified with the primer set 341F (5′-CCTACGGGNGGCWGCAG-3′) and 515R (5′-ATTCCGCGGCTGGCA-3′) [40]. The standard curves were established in triplicate from linearized plasmid serial dilutions containing between 10^9 and 10^3 16S rRNA gene copies (10^7 and 10^2 for each TRG). It was calculated directly from the concentration of extracting plasmid carrying target genes from soil and/or manure samples according to the routine method [41]. The amplification efficiency (E) was estimated from the slope of the standard curve with the following formula: $E = (10^{-1/slope}) - 1$ [42]. The efficiency of PCR between 95% and 105% was adopted for further analysis [43].