By performing a microcosm experiment mimicking fertilization, we assessed the dynamic distribution of tetracycline-resistant bacteria (TRB) and corresponding tetracycline resistance genes (TRGs) from pig manure (PM) to the fertilized soil, by culture-dependent methods and PCR detection. Cultivable TRB were most abundant in PM, followed by fertilized soil and unfertilized soil. By restriction fragment length polymorphism (RFLP) analysis, TRB were assigned to 29, 20, and 153 operational taxonomic units (OTUs) in PM, unfertilized soil, and fertilized soil, respectively. After identification, they were further grouped into 19, 12, and 62 species, showing an enhanced diversity of cultivable TRB in the soil following PM application. The proportions of potentially pathogenic TRB in fertilized soil decreased by 69.35% and 41.92% compared with PM and unfertilized soil. Bacillus cereus was likely widely distributed TRB under various environments, and Rhodococcus erythropolis and Acinetobacter sp. probably spread from PM to the soil via fertilization. Meanwhile, tetL was the most common efflux pump gene in both unfertilized and fertilized soils relative to PM; tetB(P) and tet36 were common in PM, whereas tetO was predominant in unfertilized and fertilized soil samples. Sequencing indicated that over 65% of randomly selected TRB in fertilized soil with acquired resistance derived from PM.

Due to broad-spectrum activities against a wide range of pathogenic bacteria in both humans and animals, tetracyclines (TCs) have been used in anti-infective therapy and breeding industry for many years. TCs are more frequently used for treatment and prophylaxis, and even as growth inducers, in livestock than humans, which results in the selection of resistant animal pathogens through horizontal gene transfer (HGT) by means of mobile genetic elements. The average antibiotic consumption per Chinese is nearly 10 times that of American individuals, with markedly elevated consumption by pigs in China. Consequently, animal manures possess the highest number of antibiotic resistance genes (ARGs), especially tetracycline resistance genes (TRGs). In rural China, pig manure is often applied as organic fertilizer directly to the soil without any treatment. As a major source of antibiotic pollution, it leads to large-scale soil and water pollution, harming humans through the food chain. Therefore, how to safely process pig manure before field application is of great interest in China. To achieve this, uncovering the transfer characteristics of ARGs from manure to the fertilized soil and analyzing the shift in hosts harboring TRGs are critical to understanding the vital factors affecting the biosafety of pig manure.

Multiple studies have assessed TRG distribution in various hosts by the metagenome sequencing technology. Zhu et al. found that tetQ, tetW, tetX, tet32, tetO, tetM, tetL, and tetG are most abundant in the soil. Ghosh and LaPara demonstrated that the most common genes are tetL, tetA, tetM, and tetG. Li et al. showed that tetM is central to the TRG network, and could be used as an indicator to quantitatively estimate the abundances of other TRGs. In three populations, tet32, tet40, tetO, tetQ and tetW were found to be prevalent in all gut samples, with tetQ being the most abundant. Jurado-Rabadán et al. revealed that tetM is the most common TRG in enterococci. As for TRG hosts, different results were obtained by researchers. Gao et al. found that Bacillus is the most dominant genus in tetracycline-resistant bacteria (TRB) in aquaculture environment. Huang et al. indicated...
that the majority of genera during anaerobic treatment of waste sludge are Prevotella, Caldimicrobium, Pelobacter, Pseudomonas and Clostridium with different pH levels. These findings suggest that TRG distribution varies with samples, bacterial hosts, and environmental factors. However, the dynamic occurrence and distribution of TRGs and their hosts from pig manure to the fertilized soil remain unclear, although such knowledge would help understand the actual risk of TRG transmission from pig manure.

Metagenomics can provide information about the prevalence rates of species of interest, ARGs and mobile genetic elements in various environments, and help identify novel ARGs. However, for accurate assessment of preferential ARG hosts and shift with environmental factors, the metagenomics approach seems to be unreliable, since high-throughput 16S rDNA sequencing cannot distinguish which DNA fragments come from ARB. This may lead to inaccurate associations of ARGs with their hosts. Meanwhile, using culture-dependent methods to uncover the dynamic distribution of TRGs from pig manure to the fertilized soil is feasible theoretically, although they are time-consuming. Besides, the traditional approach can probably provide information about bacterial hosts at the species level, with the possibility to further assess the evolutionary mechanism of ARGs at both the cell and gene levels.

In the present study, a microcosm experiment mimicking fertilization was performed to assess (i) the dynamic distribution of TRGs from pig manure to the fertilized soil and (ii) the preferential TRG hosts and shift during fertilization. The current findings may help elucidate the impact of pig manure on TRG distribution in the soil, also providing a basis for the further development of strategies to control TRGs.

Materials and Methods

Pig manure. Pig manure samples were collected from a pig farm with an eleven year feeding history in Qinfeng Town, Yangzhou City, which produces about 1,000 pigs yearly (pig products expanded since 2013). In normal feeds, TCs were added as production booster, and prophylactic or therapeutic agent, at a dose of 250 mg per kg feed. Daily feed consumption for each fattening pig was about 4% of body weight. Fresh pig manure excreted by adult male pigs was collected and transported to the laboratory for immediate use. By the HPLC-MS/MS method, TC amounts in manure samples were 986.3 ± 39.4 μg kg⁻¹.

Microcosm experiment. Sterile Petri dishes (150 mm × 33 mm) containing 50-gрам of pig manure, soil, and soil + pig manure, respectively, (n = 3 per group) were prepared. Soil was collected from the upper 15 cm layer from barren land in Yangzhou University, with no fertilizer applied for over ten years. The characteristics of the soil samples were: pH 6.41; soil-water ratio, 1:1; organic matter, 11.04 g kg⁻¹; cation exchange capacity, 8.96 cmol kg⁻¹. After pulverization and sieving (2 mm), soil samples were mixed evenly with pig manure specimens in different treatments mentioned above, in Petri dishes at a rate of 0.4% according to the traditional fertilization recommendations. All three treatments were placed at 25 °C and incubated for 30 days, since most organic fertilizers exhibit fertilization efficiency within 15–30 days. The moisture content of each manure sample was adjusted to 55% using sterile ddH₂O. Moisture content was derived according to the following formula: water weight (g)/dry soil weight (g) × 100%, where dry soil weight was determined after drying to constant weight at 110 °C.

Counting, screening, and identification of TRB. Ten gram samples (wet weight) were added to 90 mL of sterile dH₂O, shaken at 120 rpm, and placed at room temperature for 20 min. The flask was left for 5 min to allow soil particles to settle, followed by a ten-fold serial dilution with sterile dH₂O. A total of 100 μL of serial tenfold dilutions were plated on Luria-Bertani (LB)-TC agar medium, which comprised 1/10-strength LB 28 agar supplemented with 16 μg mL⁻¹ TC to grow cultivable TRB according to the Clinical and Laboratory Standards Institute (CLSI) document M100-S16. Agar plates were incubated at 28 °C for 24 h, followed by routine counting. From plates with around 300 colonies each, individual colonies were picked, respectively, and streaked for single colony generation on LB-TC agar medium. Bacterial strains were separately stored at −80 °C in LB broth containing 20% glycerol.

Each pure culture was grown on a LB-TC agar plate for 12–48 h depending on growth rate; then, three loops of bacterial lawns were scraped into 200 μL of sterile dH₂O, followed by incubation in a water bath at 100 °C for 10 min and centrifugation at 8000 rpm for 3 min. The resulting supernatant was stored at −20 °C as DNA template. Nearly full length 16S rRNA was amplified with primers 27f (5'-AGAGTTTGATCTGCTGAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3') by PCR. PCR was carried out in a 50 μL mixture system containing 10 μL DNA template, 0.2 mM of each dNTP, 0.4 μL of each primer, 1.25 U PrimeSTAR® HS DNA Polymerase (TaKaRa, Dalian, China), and 1 x buffer (including Mg²⁺ at 1.5 mM final concentration). Amplification was performed on an Eppendorf Mastercycler (Perkin-Elmer, Inc., Waltham, MA) under the following conditions: initial denaturation at 94 °C for 5 min; 30 cycles of 94°C for 1 min, 55 °C for 1 min, 72 °C for 1.5 min; final extension at 72 °C for 10 min. Amplification products were assessed by agarose gel electrophoresis (1% w/v agarose in Tris-Borate-EDTA buffer). The resulting PCR products were digested with the restriction enzyme Hinfl (TaKaRa, Dalian, China), separately, and distinguished according to different patterns mirrored by agarose gel electrophoresis at 1.2%. Only one randomly selected PCR product within the same Hinfl-digested fingerprint pattern was sequenced by Sangon Biotech. Co., Ltd., Shanghai, China. After comparison with the GenBank reference sequences, the obtained sequences for representative strains from different operational taxonomic units (OTUs) were deposited in GenBank using the submission tool Sequin. The accession numbers of TRB were KX981438 - KY048431 - KY048441 (duplicates were discarded, keeping only one representative strain per species). Phylogenetic trees were constructed using the neighbor-joining algorithm in MEGAS.

To further identify each strain at the species level, strains within the same genus based on 16S rRNA gene sequences were respectively subjected to identification through their biochemical and morphological properties according to the Bergey's Manual of Systematic Bacteriology.
| Primers | Targeted genes | Sequences (5′-3′) | Annealing temperature (°C) | Amplicon size (bp) | Reference |
|---------|----------------|-------------------|-----------------------------|-------------------|-----------|
| tetA-FW| tetA           | GGCGGATCTGTTCCACTCG | 61                          | 164               | [57]      |
| tetA-RV|               | AGTCGACGARYGCGCGCGG |                             |                   |           |
| tetB-FW| tetB           | TACGTGAAATTATGCTTCG | 59                          | 206               | [58]      |
| tetB-RV|               | ATACAGCTTCACAAAGCGAC |                             |                   |           |
| tetC-FW| tetC           | GCGGGATATGTCCATCCTCG | 68                          | 207               | [58]      |
| tetC-RV|               | GCAGTGAAGATCCCAGAGGCC |                             |                   |           |
| tetD-FW| tetD           | GTAATCTCAGGAGGAAGGG | 68                          | 187               | [57]      |
| tetD-RV|               | CATCCTGACAGGCCAGACGAG |                             |                   |           |
| tetE-FW| tetE           | GTTATCCCGAGTTTTGTTC | 61                          | 199               | [57]      |
| tetE-RV|               | AATACAAACCCACACTACGC |                             |                   |           |
| tetG-FW| tetG           | GCAGAGCAGGTCGCTGGGGA | 65                          | 134               | [58]      |
| tetG-RV|               | CCGCAAGAAGAACAGAAGAAG |                             |                   |           |
| tetH-FW| tetH           | CAGTGAATTTACTGCTGCAAC | 61                          | 185               | [57]      |
| tetH-RV|               | ATCCAAACGAGTGTAAGAAT |                             |                   |           |
| tetI-FW| tetI           | CGAAAGACAGACTCGCAACT  | 61                          | 184               | [57]      |
| tetI-RV|               | TCTCAGATGAGGTGGGCGG |                             |                   |           |
| tetK-FW| tetK           | TCGATAGAAGAAGCACTGGA  | 55                          | 169               | [60]      |
| tetK-RV|               | CAGCAGATCTACTCTCTT |                             |                   |           |
| tetL-FW| tetL           | TGTATAGGCTGGTCTATCCT  | 55                          | 267               | [57]      |
| tetL-RV|               | GTATCCACCAAGTGTAAGCAC |                             |                   |           |
| tetV-FW| tetV           | GCCATCGGTTTCATCTTGGCC | 65                          | 351               | [61]      |
| tetV-RV|               | CGGAACGACCTCCTCAAGACG |                             |                   |           |
| tetY-FW| tetY           | ATTTTGACTGCGAGACGGAAAAC |                             | 181               | [57]      |
| tetY-RV|               | G GCCGTGCAGCCTATATGCG |                             |                   |           |
| tetZ-FW| tetZ           | CTTCTGTGACGAGTTGGC | 68                          | 204               | [62]      |
| tetZ-RV|               | ACCCAAGCGTGTCGTCGTC |                             |                   |           |
| tetA(P)-FW| tetA(P)        | CTGGAGATGGCAGGAAAAGAG | 55                          | 676               | [60]      |
| tetA(P)-RV|              | ATATGCCCATTAAACCCCGC |                             |                   |           |
| tet30-FW| tet30          | CATCTTGTGGAGGATGCTGGA  | 68                          | 210               | [57]      |
| tet30-RV|               | AGCAGACCCCGAGGCGGAG |                             |                   |           |
| tet31-FW| tet31          | CAATCACGGCCCAGAAGAAA | 53                          | 564               | [62]      |
| tet31-RV|               | TTTGCCATTCACGTTTGTG |                             |                   |           |
| tet33-FW| tet33          | ATGGCGCTTCCGCTGAA | 54                          | 784               | [63]      |
| tet33-RV|               | GGGAAATTGGCTACGTGACAA |                             |                   |           |
| tet35-FW| tet35          | ATGCGCAGAGGGGTCCGCTCGA | 54                          | 844               |           |
| tet35-RV|               | CAACACATCAGAAACGGGTCGAA |                             |                   |           |
| tet38-FW| tet38          | ATGAATGTTGAATATATCTAAA | 42                          | 106               | [65]      |
| tet38-RV|               | TGGCCTACAAAGAATCAAT |                             |                   |           |
| tet39-FW| tet39          | CTCCTTCCTCTATTTGCTGCA  | 47                          | 701               | [66]      |
| tet39-RV|               | CACCTATCCCTTCCGACATCA |                             |                   |           |
| tet40-FW| tet40          | CCGAGGAGAAGGGACACCCACCG | 56                          | 446               |           |
| tet40-RV|               | TAAAGCGGTCGCGATAAGAC |                             |                   |           |
| tet41-FW| tet41          | ATGGCGATCAATTTGCGGCG | 55                          | 166               |           |
| tet41-RV|               | CGGCGACAGCATAGTACCGA |                             |                   |           |
| tet42-FW| tet42          | TCCTCGAGATCGCCAGACCCCT | 55                          | 128               |           |
| tet42-RV|               | ACTGCGGATCGTACGACCCA |                             |                   |           |
| tet45-FW| tet45          | GCAGGCGCCATCCTACTATTT | 63                          | 107               | [68]      |
| tet45-RV|               | TTTCCGCTTTGAGGTTTAATGCG |                             |                   |           |
| tetAB(46)-FW| tetAB(46) | GCTCTTGAGACCTGACGGGA | 55                          | 580               | This study |
| tetAB(46)-RV|             | GTTCCTGAGCTATGGCCACA |                             |                   |           |
| tet47-FW| tet47          | GCAGGTGGGCTGGAATTTAAT | 55                          | 627               | This study |
| tet47-RV|               | GACCCCCCTGCAGTTGTTA |                             |                   |           |
| tet3-FW| tet3           | CGCTAGTTTGCGAACAGACCT | 54                          | 399               | This study |
| tet3-RV|               | GTTCCTGAGCTTCCGCACT |                             |                   |           |

Continued
Table 1. PCR primers used in this study.

| Primers | Targeted genes | Sequences (5'-3') | Annealing temperature (°C) | Amplicon size (bp) | Reference |
|---------|----------------|-------------------|-----------------------------|------------------|-----------|
| otrB-FW | otrB           | CCGACATCTACGGGCGCAAGC | 55                           | 947              | [68]      |
| otrB-RV |               |                   |                              |                  |           |
| otrC-FW | otrC           | ATGAAGTTCCGGCGGCGAAATGNA | 55                           | 1860             | [70]      |
| otrC-RV |               |                   |                              |                  |           |
| tetM-FW | tetM           | ACAGAAACGTATTATATATAAC | 55                           | 171              | [68]      |
| tetM-RV |               |                   |                              |                  |           |
| tetO-FW | tetO           | ACAGGARAGTTTATTTATGATGCC | 60                           | 171              | [68]      |
| tetO-RV |               |                   |                              |                  |           |
| tetQ-FW | tetQ           | AGAATCTGCGTTGCGCGAGTG | 56                           | 169              | [68]      |
| tetQ-RV |               |                   |                              |                  |           |
| tetS-FW | tetS           | GAAAGGTCTACTATACAGTAGC | 50                           | 169              | [68]      |
| tetS-RV |               |                   |                              |                  |           |
| tetT-FW | tetT           | AAGGTATATATATATAAAGTG  | 46                           | 169              | [71]      |
| tetT-RV |               |                   |                              |                  |           |
| tetW-FW | tetW           | GAGAGCTGCTATATCCGCGG    | 64                           | 168              | [68]      |
| tetW-RV |               |                   |                              |                  |           |
| tetB(P)-FW | tetB(P)             | AAAAACTTATTATTATTATAGTG | 46                           | 169              | [68]      |
| tetB(P)-RV |                |                   |                              |                  |           |
| tet32-FW | tet32           | GAACAGATGCTGCTTCTT     | 57                           | 620              | [72]      |
| tet32-RV |               |                   |                              |                  |           |
| tet36-FW | tet36           | TTTCTGCGACAGGTAAGACGCC | 57                           | 250              | [73]      |
| tet36-RV |               |                   |                              |                  |           |
| tet44-FW | tet44           | AAAATATACACATGGTAGATTGTGTGCTCA | 56                           | 1927             | [74]      |
| tet44-RV |               |                   |                              |                  |           |
| tet34-FW | tet34           | ATACGGGGTGGCAAACTTCA   | 53                           | 729              | [63]      |
| tet34-RV |               |                   |                              |                  |           |
| tet37-FW | tet37           | ATGGTTCGATATTACCTTACAC | 45                           | 177              | [76]      |
| tet37-RV |               |                   |                              |                  |           |
| tetU-FW | tetU           | ATGCAGCTAAGACGCGGCG    | 54                           | 317              | [67]      |
| tetU-RV |               |                   |                              |                  |           |

Figure 1. Correlation between the percentage of TRGs in cultivable TRB and TRG abundance obtained by the culture-independent approach. To avoid excessive differences in TRGs, the latter were ranked in each treatment.
PCR detection of TRGs in TRB. PCR was employed to qualitatively assess currently known TRGs in TRB. Both genomic and plasmid DNAs were extracted with corresponding kits (Tiangen Biotech, Beijing) and mixed evenly. The mixed DNA was amplified for 44 target TRGs, including the 29 efflux pump genes tetA, tetB, tetC, tetD, tetE, tetG, tetH, tetI, tetJ, tetK, tetL, tetM, tetN, tetQ, tetR, tetS, tetT, tetU, tetV, tetW, and tetZ; 11 ribosomal protection protein (RPP) coding genes tetM, tetO, tetQ, tetS, tetT, tetW, tetB(P), tet32, tet36, tet44, and tet48; 3 tetracycline-modifying enzyme genes tetX, tet34, and tet37; and tetU gene with unknown function. PCR amplification was performed in a 25 μl reaction system containing 2.5 μl of 10× PCR buffer (including Mg2+ at a final concentration of 1.5 mM), 0.125 μl of each primer (30 μM) listed in Tables 1, 2 μl of DNA template, 0.25 μl of each deoxynucleoside triphosphate (80 mM), and 0.1 μl of Taq DNA polymerase (Takara, Dalian, China). Amplification was performed on an Eppendorf Mastercycler (Perkin-Elmer Inc., Waltham, MA) under the following conditions: initial denaturation at 94 °C for 4 min; 35 cycles of 94 °C for 5 s, different annealing temperatures (listed in Table 1) for 45 s, 72 °C for 1 min; final extension at 72 °C for 6 min. Amplification products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light.

To confirm the TRGs base on size, five randomly selected bands for a particular TRG were excised from the agarose gel, followed by DNA recovery with a specific kit. The purified DNA was cloned into the pMD18-T vector (Takara Bio Inc.) and transformed into chemically competent E. coli DH5α. The extracted plasmid DNA from a positive clone was sequenced with universal primers by Sangon Biotech. Co., Ltd., Shanghai, China. After sequence comparison with BLAST, the confirmed PCR product was loaded on the gel as the positive control to verify other PCR products obtained for the same TRG.

Correlation between the TRG percentage in cultivable TRB and TRG abundance obtained with the culture-independent approach. To assess if the culture-dependent method adopted in this study was reliable, the percentage of TRGs in TRB and TRG abundance obtained by real-time quantitative PCR (q-PCR) approach were assessed.

Total microbial DNA was extracted from manure, soil, and soil + pig manure samples with Power-Soil™ DNA Isolation Kit (MO BIO Laboratories Inc., CA, USA) according to the manufacturer’s instructions. Six TRGs, namely tetB, tetC, tetM, tetO, tetT, and tetZ genes, were amplified with primers described in our previous work23. PCR was performed on a Bio-Rad MiniOpticon (Bio-Rad Laboratories, CA, USA) with SYBR Green I for estimating the copy numbers of TRGs. A total of 20 μl reaction system containing 10 μl of iTaq Universal SYBR Green Supermix, 0.4 mM of each primer, and 10 ng of template DNA was set up. The amplification 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 the tetB, tetC, tetM, tetO, tetT, and tetZ genes, respectively), and the subsequent dissociation curve generation. Data were analyzed for target genes from soil and/or manure samples as previously described32. Amplification efficiency (E) was estimated from the slope of the standard curve with the following formula: $E = (10^{−1/\text{slope}}) − 1$33. PCR efficiency between 95% and 105% was adopted for further analysis34.

Data analysis. Raw data were imported into Excel for analysis. Network visualization was performed on the interactive platform Cytoscape (version 3.2.0). Other graphs were obtained using Sigma Plot for Windows Version 10.0 (Systat Software, San Jose, CA, USA).

Results

Correlation between the percentage of TRGs in cultivable TRB and TRG abundance obtained by the q-PCR approach. It is necessary to assess whether the culture-based method adopted in this study is feasible. We therefore evaluated the correlation between the percentages of six randomly selected TRGs in cultivable TRB and their abundance levels obtained by the q-PCR approach. To avoid large differences in TRG abundance levels, the data obtained by the two methods were ranked and shown in Fig. 1. A good linear relationship was observed, indicating the reliability of the method used in this work.

Cultivable TRB. Although cultivable TRB in pig manure were about four and three log units higher than those in unfertilized soil and soil + pig manure samples, OTU and species numbers were lower than those of soil + pig manure treatment (Table 2). Among the three treatments, the indices of cultivable TRB in the unfertilized soil were ranked lowest. These findings indicated that (1) relatively high abundance and low diversity of cultivable TRB were found in pig manure, and (2) cultivable TRB in the soil could be greatly enhanced by pig manure application.

| Treatment        | Cultivable TRB (lg cfu/g dry sample) | OTUs numbers | Species numbers | Percentage of possible pathogen (%) |
|------------------|-------------------------------------|--------------|-----------------|------------------------------------|
| Pig manure       | 8.12a                               | 29           | 19              | 47.37 (9/19)                       |
| Soil             | 3.98b                               | 20           | 12              | 25.00 (3/12)                       |
| Soil + Pig manure| 5.21c                               | 153          | 62              | 14.52 (9/62)                       |

Table 2. Cultivable TRB and species in the three samples. OUT numbers were obtained by comparison of HinfI-digested fingerprint patterns; species numbers were obtained by 16S rRNA gene sequencing combined with biochemical and morphological properties. *Means within columns followed by different letters are significantly different (Duncan’s test, $p < 0.05$).
The succession in cultivable TRB at the species level from pig manure to fertilized soil is shown in Fig. 2. Specific species in pig manure, soil, and soil + pig manure accounted for 19.5%, 4.9%, and 52% of all species, respectively. *Bacillus cereus* was present in all three samples, and represented relatively abundant TRB in the environment. Most species in pig manure were not present in the fertilized soil, which indicated that other factors such as nutrients played stimulatory roles in the enhancement of bacterial species. Seven species, including *Chryseobacterium lathyri*, *Rhodococcus equi*, *Microbacterium sp.*, and *Pseudomonas fragi*, were found in both unfertilized and fertilized soils, suggesting that they may be stubborn soil species which are hard to control. *R. erythropolis* and *Acinetobacter sp.* were probably spread from pig manure to the soil via fertilization, and more attention should to be paid to these species.

**Frequency of the detected tetracycline resistance determinants.** In cultivable TRB derived from the three samples, except *tetY, tet38, tet45, tet44*, and *tet34*, the remaining 39 TRGs were all found at different frequencies. In general, the detected species possessed efflux pump genes in all three samples, with most of

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**Figure 2.** Venn diagram of shared TRB at the species level among the three samples. Species highlighted in red are potential pathogens.

**Figure 3.** Percentages of the four TRG groups in TRB from the three samples.
them having multiple efflux pump genes (Fig. 3). For example, *Arthrobacter protophormiae* (accession number KY048441), *Stenotrophomonas koreensis* (accession number KY048438), and *Acinetobacter* sp. (accession number KY048432) had 13 such genes. The frequencies of RPP and enzymatic modification genes were similar in each sample, and these two TRG groups in the fertilized soil were about 50% lower than in pig manure and unfertilized soil samples. The TRG with unknown function (*tetU*) showed highest frequency in pig manure, followed by soil and fertilized soil samples.

Of the efflux pump genes, *tetB*, *tetL*, and *tetZ* were the most common TRGs in pig manure, with frequencies 94.74%, 84.21%, and 68.42%, respectively; *tetL* (83.33%), *tetB* (75.00%), and *tetA* (66.67%) showed the highest frequencies in soil sample, and the top three efflux pump genes in the fertilized soil were *tetL* (82.26%), *tetA*...
(74.19%), and tetB (69.35%) (Fig. 4). As for RPP genes, tetB(P), tet36, tetM, and tetO were found at more than 30%, while in unfertilized and fertilized soil samples tetO absolutely had the highest frequency. Meanwhile, tet37 and tetX were both detected in pig manure at frequencies of 47.37% and 36.84%, respectively, while only the tetX gene was found in unfertilized and fertilized soil samples at frequencies 75.00% and 50.00%, respectively.

**Preferential hosts for different TRG groups.** The networks of efflux pump genes and their hosts are shown in Fig. 5. The most complex network of TRGs and their hosts was obtained in the fertilized soil, followed...
by pig manure and normal soil. This finding indicated that pig manure application promoted the expression of efflux pump genes among diverse bacterial hosts. From pig manure and normal soil to fertilized soil, preferential hosts for efflux pump genes were changed from *Stenotrophomonas koreensis* (9 efflux pump genes), *Providencia vermicola* (9), *A. protophomiae* (8), *Acinetobacter sp.* (8), *Paenibacillus lautus* (10), *Sphingobacterium anhuiense* (10), *P. fragi* (7), and *Rhodococcus equi* (6) to *Variovorax paradoxus* (9), *Achromobacter mucicolens* (9), *Acinetobacter sp.* (8), *P. frederiksbergensis* (9), *Bacillus sp., et al.* (7). This finding suggested that *Acinetobacter sp.* was probably spread with fertilization, and changes in other preferential hosts for efflux pump genes in the fertilized soil might be stimulated by pig manure.

The preferential hosts for RPP genes were also changed obviously with pig manure application (Fig. 6). In unfertilized soil samples, all species had only *tetO* as RPP gene, while two groups of networks were distinguished.

**Table 3.** GC-contents of genomic *tetL* in different hosts. *tetL* was amplified using the primer pair *tetL*-FW (5′-GTGTTGCGGCTATATTCG-3′) and *tetL*-RV (5′-GTGAAMGRWAGCCCACCTAA-3′).

| Host                          | GC-content of *tetL* | Genomic GC-content of TRB | Difference over 10% |
|-------------------------------|----------------------|---------------------------|---------------------|
| *Bacillus thuringiensis* 8    | 35.3                 | 35.0                      | No                  |
| *Oceanobacillus oncorhynchi* 32| 34.7                 | 39.3                      | Yes                 |
| *Rhodococcus erythropolis* 45 | 34.1                 | 62.3                      | Yes                 |
| *Bacillus cereus* 91          | 34.0                 | 35.2                      | No                  |
| *Bacillus aquimaris* 113      | 34.9                 | 43.3                      | Yes                 |
| *Myroides odoratimimus* 122   | 34.4                 | 34.1                      | No                  |
| *Psychrobacter palmonis* 135  | 34.2                 | 42.8                      | Yes                 |
| *Myroides odoratimimus* 140   | 33.9                 | 34.1                      | No                  |
| *Rhodococcus canchipurensis* 199| 34.0                 | 65.3                      | Yes                 |
| *Alcaligenes faecalis* 229    | 33.8                 | 56.7                      | Yes                 |
| *Stenotrophomonas koreensis* EMB15| 33.9                 | 66.1                      | Yes                 |

**Table 4.** GC-contents of genomic *tetX* in different hosts. *tetX* was amplified using the primer pair *tetX*-FW (5′-ATGACAAATGCCGAATAGATACAGACA-3′) and *tetX*-RV (5′-CAATTGCTGAAACGTAAAGTC-3′).

| Host                          | GC-content of *tetX* | Genomic GC-content of TRB | Difference over 10% |
|-------------------------------|----------------------|---------------------------|---------------------|
| *Pseudomonas caeni* 14        | 38.5                 | 48.3                      | Yes                 |
| *Pedobacter busanensis* 77    | 38.5                 | 38.7                      | No                  |
| *Psychrobacter palmonis* 135  | 38.3                 | 42.8                      | Yes                 |
| *Lyso bacter antibioticus* 152| 38.3                 | 67.0                      | Yes                 |
| *Facklamia tabacinaalis* 168  | 38.5                 | 38.9                      | No                  |
| *Wautersiella falsenii* EMB5   | 38.7                 | 32.1                      | Yes                 |

**Figure 8.** Network of a TRG with unknown function and its hosts isolated from pig manure (A) and soil + pig manure (B).
in pig manure and fertilized soil specimens. In the fertilized soil, increases of *B. flexus*, *Streptomyces filamentosus*, *V. boronicumulans*, *S. castaneus*, *et al.* may be stimulated rather than introduced by pig manure. *Acinetobacter* sp. may also be the host for tetracycline-modifying enzyme genes introduced by pig manure (Fig. 7). *B. cereus* was a common host for genes in all three treatments. Except for *Microbacterium* sp. and *R. equi*, other species were possibly stimulated by pig manure. Besides, the hosts of the unknown TRG *tetU* seemed to be also induced by pig manure (Fig. 8).

**Discussion**

The negative effects of pig manure on TRG spread to the soil require special attention. Multiple studies have reported the high abundance and diversity of TRGs and/or TRB in pig manure and commercial organic fertilizers44,22,25,36. However, this study found that cultivable TRB in fertilized soil were three times more diverse than in pig manure and soil (Table 2), indicating that pig manure application does not only enhance TRG abundance but also, more importantly, could increase the diversity of cultivable TRB in the soil. This undoubtedly intensifies the negative effects of pig manure on the spread of TRGs to the soil. Using a metagenomics approach, Udikovic-Kolic *et al.* showed that manure-treated soil has less phylogenetic diversity of bacteria compared with NPK-treated soil57. Although bacterial diversity in fertilized and unfertilized soils was not assessed in the current study, it can be inferred that larger proportions of bacteria were tetracycline resistant in the fertilized soil compared with the untreated soil, with many of them harboring proto-resistance or silent resistance genes39, which change into the tetracycline-resistant type following manure application.

Intriguingly, pig manure application did not increase the percentage of pathogenic TRB in the soil (Table 2, Fig. 2); meanwhile, the diversity of pathogenic TRB decreased from 47.37% (pig manure) and 25.00% (untreated soil) to 14.52% (fertilized soil). On the one hand, some TRB with antagonistic effects became predominant following pig manure application, and may be capable of inhibiting sensitive pathogenic bacteria. For example, *B. amyloliquefaciens*40, *P. fluorescens*38, *B. thuringiensis*34, *S. tanashiensis*42, *P. vancomurensis*43. *Chryseobacterium wanjaense*44, *et al.* are known for such activities. In addition, most pathogenic TRB (except *B. cereus*), such as *Myroides odoratimimus* and *Alcaligenes faecalis*, in pig manure may be more adapted to the environment than untreated or fertilized soil, since they are common in the gut environment45,46.

It can be inferred that *R. erythropolis* and *Acinetobacter* sp. were probably spread from pig manure to soil via fertilization, and more attention should be paid to these species. *R. erythropolis* can cause bloodstream infection47, and was firstly detected in pig manure. The high adaptability in distinct and even extreme environments of this TRB has been reported by many studies48,49; this may be the reason for its wide distribution. *Acinetobacter* sp. in this study was not accurately identified at the species level, but the relatively high amount of TRGs as well as the wide distribution traits in this species also requires attention. Besides, *B. cereus*, which possessed around 5 TRGs, was found in all three samples. *B. cereus* is an opportunistic pathogen capable of causing food poisoning50; however, it is often isolated for its potential to promote plant growth, and has been developed for commercial use51,52. Therefore, attention should be paid when using bio-agents containing this bacterium.

**TRGs have diverse and distinct hosts between pig manure and the fertilized soil, with a high risk of spreading TRGs via pig manure application.** To date, little is known about the changes of TRGs from pig manure to the soil. As shown above, *tetL* was the most common efflux pump gene in both untreated and fertilized soils versus pig manure, which is partly consistent with Peng *et al.*53. Besides, *tet42* and *tetK* were most common in pig manure followed by fertilized soil and untreated soil, suggesting that they could be introduced into the soil via fertilization. Differences of *tetA* and *tet33* in the three samples were also obvious, indicating that the four genes *tet42*, *tetK*, *tetA*, and *tet33* could be used as indicators for monitoring efflux pump genes in TRGs among various treatments. However, further investigation is required since (1) the above data were obtained by (2) the hosts of the unknown TRG *tetU* seemed to be also induced by pig manure (Fig. 8).
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Author Contributions
Y.K. and J.H. planned the project and wrote the manuscript. Q.L. and Z.Y. managed and performed all experiments. H.Z. and M.S. helped with material treatment. C.B. and L.J. helped analyze the data. All authors discussed the results and reviewed the manuscript.
Additional Information

Competing Interests: The authors declare that they have no competing interests.

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