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Attenuation of veterinary antibiotics in full-scale vermicomposting of swine manure via the housefly larvae (*Musca domestica*)

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Animal waste from concentrated swine farms is widely considered to be a source of environmental pollution, and the introduction of veterinary antibiotics in animal manure to ecosystems is rapidly becoming a major public health concern. A housefly larvae (*Musca domestica*) vermireactor has been increasingly adopted for swine manure value-added bioconversion and pollution control, but few studies have investigated its efficiency on antibiotic attenuation during manure vermicomposting. In this study we explored the capacity and related attenuation mechanisms of antibiotic degradation and its linkage with waste reduction by field sampling during a typical cycle (6 days) of full-scale larvae manure vermicomposting. Nine antibiotics were dramatically removed during the 6-day vermicomposting process, including tetracyclines, sulfonamides, and fluoroquinolones. Of these, oxytetracycline and ciprofloxacin exhibited the greater reduction rate of 23.8 and 32.9 mg m⁻², respectively. Environmental temperature, pH, and total phosphorus were negatively linked to the level of residual antibiotics, while organic matter, total Kjeldahl nitrogen, microbial respiration intensity, and moisture exhibited a positive effect. Pyrosequencing data revealed that the dominant phyla related to *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* accelerated manure biodegradation likely through enzyme catalytic reactions, which may enhance antibiotic attenuation during vermicomposting.

Large quantities of raw manure are produced in swine farms throughout the world today as a result of the development of concentrated animal feeding operations¹. Animal manure is traditionally applied to farmlands as a bio-fertilizer to provide essential nutrients, which may cause environmental pollution due to the release of pathogens and excess nutrients into bodies of water resulting in the public health risk of eutrophication². Best management practices, such as composting and anaerobic digestion, have been proposed for animal waste prior to land application in order to mitigate health and environmental risks²; however, these options cannot be broadly applied, particularly in developing countries, due to limitations in efficiency and cost. Thus, there is an urgent need to develop both economically and environmentally sustainable technologies for swine manure management.

Some studies have exploited the ability of several species of diptera to grow naturally on animal waste as a substrate^{3–6}, and diptera larvae have been used instead of earthworms^{3,4,7,8} to vermicompost animal and/or agroindustrial waste. In particular, the housefly (*Musca domestica*) was chosen for vermicomposting of animal manure^{7,9} due to its short interval of generation (5–7 days) and high reproductive capacity^{1,5,7}. While previous studies have validated the overall process and economic feasibility of housefly larvae-vermicomposting^{1,5,7}, little is known about its efficiency in the degradation and removal of contaminants, such as veterinary antibiotics, in animal manure.

Current livestock production uses large amounts of antibiotics as growth promoters and therapeutic agents¹⁰. Tetracyclines (TCs), sulfonamides (SAs), and quinolones (QLs) are three classes of broad-spectrum antibiotics



widely used in the livestock industry in China^{11,12}. It is estimated that approximately 75% of the antibiotics used are not absorbed and are instead excreted in waste¹⁰. Antibiotic residue in manure can reach the soil-water ecosystem by run-off and/or leaching, creating one of the main sources of terrestrial pharmaceutical pollution and is of major concern to environmental and human health^{13–15}. A number of studies have demonstrated that incubating animal manure under a variety of composting conditions results in a significant decrease in the concentration of extractable antibiotics^{16–18}, primarily due to abiotic degradation and sorption¹⁸. Compared to conventional composting, housefly larvae-vermicomposting has been shown to have a superior biodegradation capacity due to the presence of the unique larvae-microorganism complexes^{5,7}. Therefore, housefly larvae-vermicomposting could be potentially effective in the degradation of veterinary antibiotics in animal manure.

A full-scale operation of swine manure vermicomposting with housefly larvae has been under operation since 2008 in HangZhou, China. In this study, the attenuation of antibiotics as well as biochemical and microbial characteristics of the vermicomposting process was monitored within a typical generation interval (6 days) with the following objectives: 1) assess the effectiveness of housefly larvae-vermicomposting in the degradation of antibiotic residues in swine

manure; 2) evaluate the linkages between the degradation of veterinary antibiotics and reduction in manure mass; and 3) gain insight into the degradation processes responsible for antibiotic attenuation.

Results

Dynamics of antibiotic attenuation during housefly larvae vermicomposting. Vermicomposting of swine manure had a significant impact on the concentration of all antibiotics tested, while few changes occurred under the ambient conditions with no larvae inoculation (Table S1). The reduction in two classes of antibiotics, TCs and SAs, was consistent throughout vermicomposting (Fig. 1). Among TCs and SAs, levels of oxytetracycline (OTC), chlortetracycline (CTC), and sulfadiazine (SDZ) exhibited the greatest reduction across the 6-day bioconversion with average rates of 23.8 mg m⁻², 8.34 mg m⁻², and 4.09 mg m⁻² within vermicomposting (Table 1). These values represented cumulative removals of 68.6%, 69.8%, and 72.3% of the total antibiotic mass from raw manure input, respectively (Fig. 1). Some rebound in antibiotic content was observed on day 6 (Table S1) due to the addition of manure solids before larvae harvesting, which caused some decrease in the daily reduction rates (Table 1). The trend in the rate reduction across the class of QLs was not consistent, with the

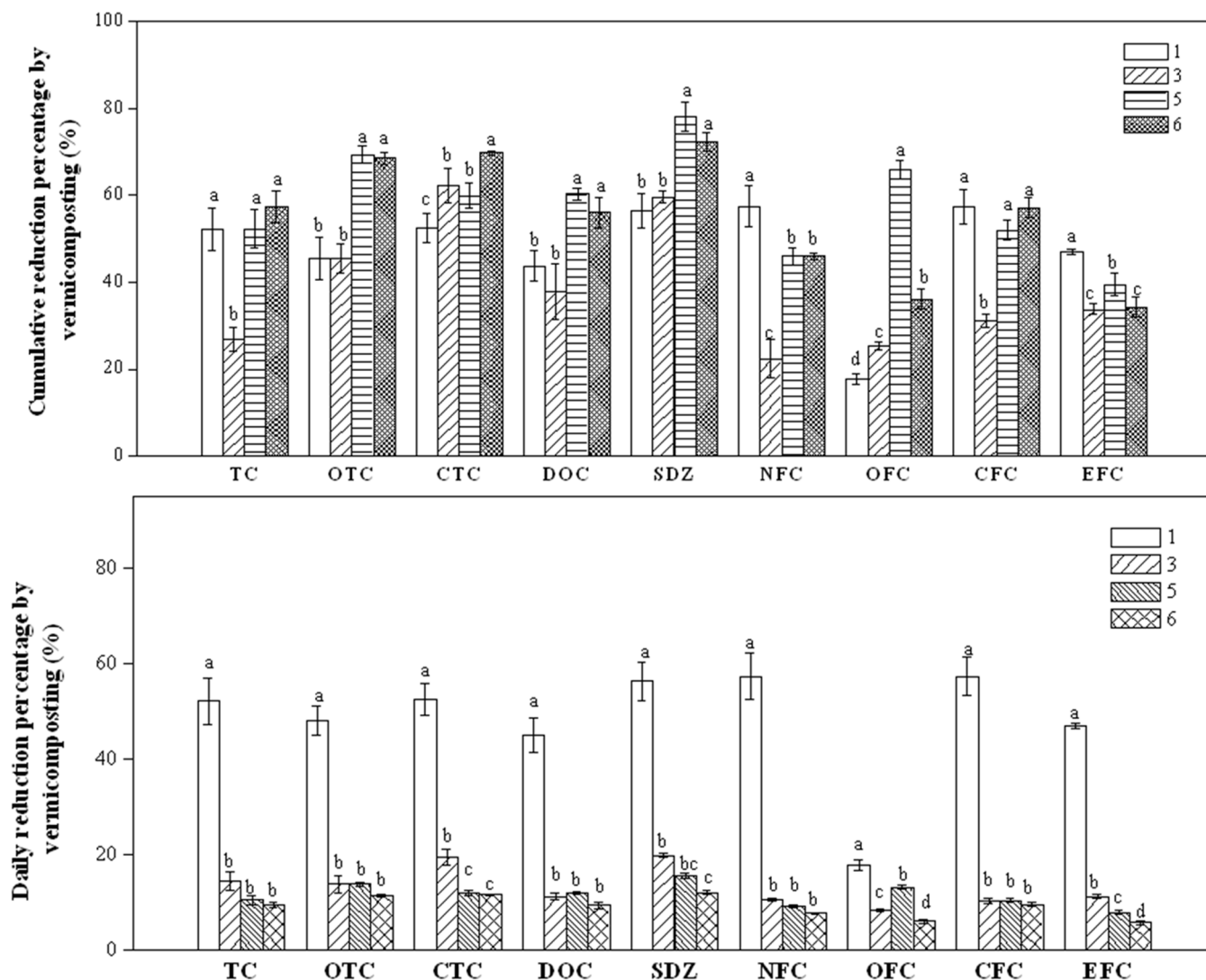


Figure 1 | Cumulative and daily antibiotic reduction in vermicompost after 1 (T-1), 3 (T-3), 5 (T-5), and 6 days (T-6) during swine manure larvae vermicomposting. Nine common antibiotics were detected: tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC), doxycycline (DOC), sulfadiazine (SDZ), norfloxacin (NFC), ofloxacin (OFC), ciprofloxacin (CFC), and enrofloxacin (EFC).



Table 1 | Cumulative and daily reduction rates of antibiotics after 1 (T-1), 3 (T-3), 5 (T-5), and 6 days (T-6) during swine manure vermicomposting using the housefly larvae

	TC	OTC	CTC	DOC	SDZ	NFC	OFC	CFC	EFC
Cumulative reduction rate (mg m⁻²)									
T-1	0.186 ± 0.005c	5.23 ± 0.34 c	3.67 ± 0.03d	0.204 ± 0.014d	0.945 ± 0.30d	0.476 ± 0.031b	0.119 ± 0.004d	10.7 ± 0.5d	3.20 ± 0.40d
T-3	0.452 ± 0.027b	14.5 ± 1.0b	6.65 ± 0.43c	0.332 ± 0.016c	2.74 ± 0.06c	0.356 ± 0.017c	0.523 ± 0.017c	16.8 ± 0.5c	6.57 ± 0.24c
T-5	0.542 ± 0.046a	23.7 ± 0.4a	7.62 ± 0.25b	0.644 ± 0.010b	3.82 ± 0.03b	1.17 ± 0.04a	1.51 ± 0.05a	29.9 ± 1.4b	8.58 ± 0.56a
T-6	0.595 ± 0.037a	23.8 ± 0.5a	8.34 ± 0.06a	0.713 ± 0.011a	4.09 ± 0.12a	1.17 ± 0.02a	0.857 ± 0.036b	32.9 ± 1.4a	7.92 ± 0.18b
Daily reduction rate (mg m⁻²d⁻¹)									
T-1	0.194 ± 0.007a	5.24 ± 0.34a	3.67 ± 0.00a	0.216 ± 0.019a	0.935 ± 0.033a	0.476 ± 0.031a	0.123 ± 0.051c	10.9 ± 0.6a	3.20 ± 0.40a
T-3	0.145 ± 0.020b	5.26 ± 0.15a	2.20 ± 0.18b	0.104 ± 0.010c	0.913 ± 0.020a	0.119 ± 0.007d	0.174 ± 0.006b	5.36 ± 0.26b	2.19 ± 0.08b
T-5	0.108 ± 0.009c	4.82 ± 0.14a	1.43 ± 0.67c	0.144 ± 0.003b	0.808 ± 0.027b	0.238 ± 0.011b	0.298 ± 0.012a	5.94 ± 0.27b	1.72 ± 0.11c
T-6	0.099 ± 0.006c	3.92 ± 0.08b	1.39 ± 0.10c	0.112 ± 0.007c	0.630 ± 0.011c	0.196 ± 0.003bc	0.139 ± 0.008c	5.49 ± 0.22b	1.33 ± 0.33d

exception of ofloxacin (OFC), and averaged 1.17 mg m⁻² and 32.9 mg m⁻² for norfloxacin (NFC) and ciprofloxacin (CFC), respectively (Table 1), while the cumulative removal reached 34.3–58.1%. All nine antibiotics exhibited rapid reduction during vermicomposting, with daily reduction percentages ranging from 7.8% to 57.4% (Fig. 1). However, the daily rate of reduction for all antibiotics declined over time (Table 1).

Dynamics of swine manure reduction during housefly larvae vermicomposting. Under the semi-continuous feeding mode, the content of organic matter (OM) was clearly reduced from 91.4% (dry weight) in raw manure to 83.7% in the vermicompost during one-week of vermicomposting (Fig. S1). Moreover, the average content of total Kjeldahl nitrogen (TKN) decreased from 3.52% to 2.61%, while levels of total phosphorus (TP) and total potassium (TK) remained the same with some minor fluctuations over time. A cumulative reduction rate of 35.5 kg m⁻² for total manure mass was achieved following the 6-day vermicomposting, leaving 17.8 kg m⁻² of vermicompost at the end of the process representing one-third of the total input of raw manure (Fig. 2a). The moisture content of vermicompost dropped continuously from 81.4% to 50.9% (Fig. S2), with a maximum reduction rate of 5.1 kg m⁻² d⁻¹ at T-6 (Fig. 2b) and a cumulative reduction rate of 34.3 kg m⁻² (Fig. 2a). The pH of the vermicompost increased from 6.67 to 8.46 during vermicomposting with a sharp increase at T-3. In addition, the temperature inside the vermireactor ranged between 30°C and 48°C, and increased gradually before day 5 with an apparent drop around the completion of vermicomposting on day 6 (Fig. 2c).

The levels of OM and TKN showed a significant decrease during vermicomposting ($p < 0.05$), but the levels of TP and TK were not significantly reduced (Fig. 3). The cumulative removal rates for OM, TKN, TP, and TK were 4183 g m⁻², 211 g m⁻², 82 g m⁻², and 41 g m⁻², respectively (Fig. 3, $p < 0.05$). The average daily removal rates for OM, TKN, TP, and TK followed the same trend, averaging 697 g m⁻² d⁻¹, 35 g m⁻² d⁻¹, 14 g m⁻² d⁻¹, and 7 g m⁻² d⁻¹, respectively (Fig. 3, $p < 0.05$). There were also considerable changes in the biochemical features of the vermicompost (Table 2). The activity of β -1,4-glucosidase (β G) continuously increased, reaching 525 mmol h⁻¹ g⁻¹ at T-5, and then decreased to 369 mmol h⁻¹ g⁻¹ at T-6. Cellobiohydrolase (CBH) activity steadily decreased over time from the highest level of 758 mmol h⁻¹ g⁻¹ at T-1. In addition, housefly larvae caused a significant reduction in the activities of β -1,4-N-acetylglucosaminidase (NAG) and acid phosphatase (AP), which reached 207 mmol h⁻¹ g⁻¹ and 25.9 mmol h⁻¹ g⁻¹ after the 6th day of vermicomposting, respectively (Table 2). A notable increase was observed in the tested enzymes (except β G), with increases in activity by 18% (CBH) to 86% (AP) within the first 3 days compared to control; however, the opposite trend also occurred, with drops by 15% (CBH) to 61% (NAG) at the end of vermicomposting (T-6). In addition, microbial cumulative mineralization was consistently suppressed over the course of vermicomposting, with the respiration rate dropping from 4768 μ g g⁻¹ at C-0 to 2016 μ g g⁻¹ at T-6 (Fig. 2d).

Response of bacterial diversity during manure larvae vermicomposting. The 16S rRNA 454 sequencing identified bacterial operational taxonomic units (OTUs) from 17 phyla (*Firmicutes*, *Bacteroidetes*, *unclassified*, *Proteobacteria*, *Tenericutes*, *Spirochaetes*, *Actinobacteria*, *Verrucomicrobia*, *Fibrobacteres*, *TM7*, *Synergistetes*, *Fusobacteria*, *Lentisphaerae*, *Cyanobacteria*, *Chloroplast*, *OD1*, and *SRI1*) in swine manure (C-0) and vermicompost at T-3 and T-6. We selected 9 phyla with the highest relative abundance in order to explore the structural transition of the bacterial community. The predominant populations, which were the *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* phyla, changed from 40.5% to 52.9%, 25.9% to 15.6%, and 10.0% to 15.7% during vermicomposting, respectively (Fig. 4). The total number of bacterial OTUs also decreased after vermicomposting: *Verrucomicrobia*, *Fibrobacteres*, and *Spirochaetes*

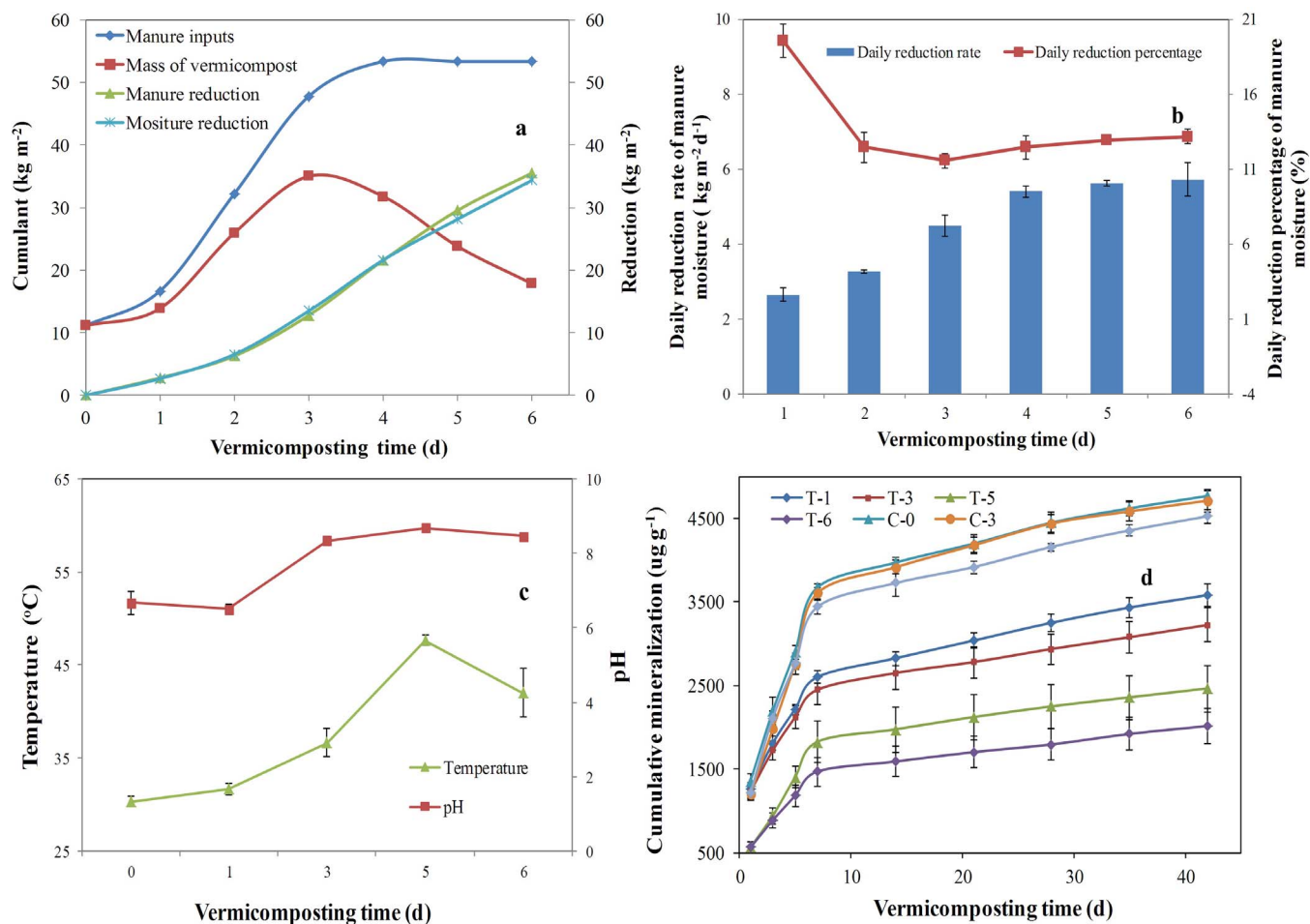


Figure 2 | Dynamics of cumulative input of raw manure, mass of vermicompost, and reductions in raw manure and moisture (a), daily reduction rate and percentage of moisture (b), temperature and pH changes (c), and intensity of mineralization (d) during swine manure larvae vermicomposting.

were absent from vermicompost at T-6, while the regnant phyla *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* OTUs declined by 53.9%, 78.7%, and 44.9%, respectively (Fig. S3).

The linkage between antibiotic dynamics and manure/vermicompost features. The relationship between the physico-biochemical properties of vermicompost and the concentration of residual antibiotics was statistically evaluated by redundancy analysis (RDA) (Fig. 5). Axis 1 explained 51.9% of the variance contributed by the differences in characteristics between raw manure and vermicompost, while axis 2 explained 6.9% of the variance from sampling time. Among the environmental and physico-biochemical variables, temperature, pH, and TP were negatively linked to the level of residual antibiotics, while OM, TKN, respiration intensity, and moisture had a positive effect. Enzymatic activities were not directly linked to antibiotic concentrations. Furthermore, the control and treatment samples were differentiated by RDA (Fig. 6). The *Verrucomicrobia*, *Fibrobacteres*, and *Fusobacteria* OTUs were significantly negatively linked to antibiotic levels of vermicompost at T-3 and T-6, including other bacterial phyla. The different characteristics between raw manure and vermicompost contributed to 66.3% of the variance for axis 1, while the variances from sampling time contributed 17.3% to axis 2.

Discussion

In this study, a notable reduction in antibiotic concentration induced by the larvae-microorganism complexes was observed, with cumulative reductions ranging from 34% to 72% for TCs, SAs, and QLs

(Fig. 1). Previous studies have reported the removal of veterinary antibiotics through traditional composting of animal manure. A 99% reduction in salinomycin concentration was achieved over 38 days¹⁴, while more than 99% of NFCs were removed during 40 days of broiler manure composting¹³. Reductions of 54% to 76% were observed for monensin and tylosin during a period of 22–35 days¹⁹. In comparison, the vermicomposting process tested in this study achieved a reduction of up to 70% within 6 days (Table 1, Fig. 1), which is a much shorter time period than that required for traditional composting processes. The shorter duration required for vermicomposting provides a significant advantage of process efficiency for the control of antibiotics in animal manure.

In recent years, many researchers have focused on the attenuation of veterinary antibiotics using various matrix and management technologies. At the environmentally relevant concentrations tested in activated sludge, biodegradation and adsorption were the major removal routes for these antibiotics^{20,21}. Since antibiotics are generally introduced from livestock into the environment, hydrolysis is an important degradation pathway²². Due to the susceptibility to photodegradation, other studies have demonstrated that photolysis is another abiotic transformation process¹⁰. In composting, the abiotic degradation conditions, such as temperature and degradation via epimerization, are likely to control the dissipation of antibiotics^{16,17}. In our study, aside from common environmental factors such as temperature and pH (Fig. 5), which positively contribute to antibiotic biodegradation and transformation^{10,23}, the chemical/biochemical factors linked to the reduction of swine manure were also correlated

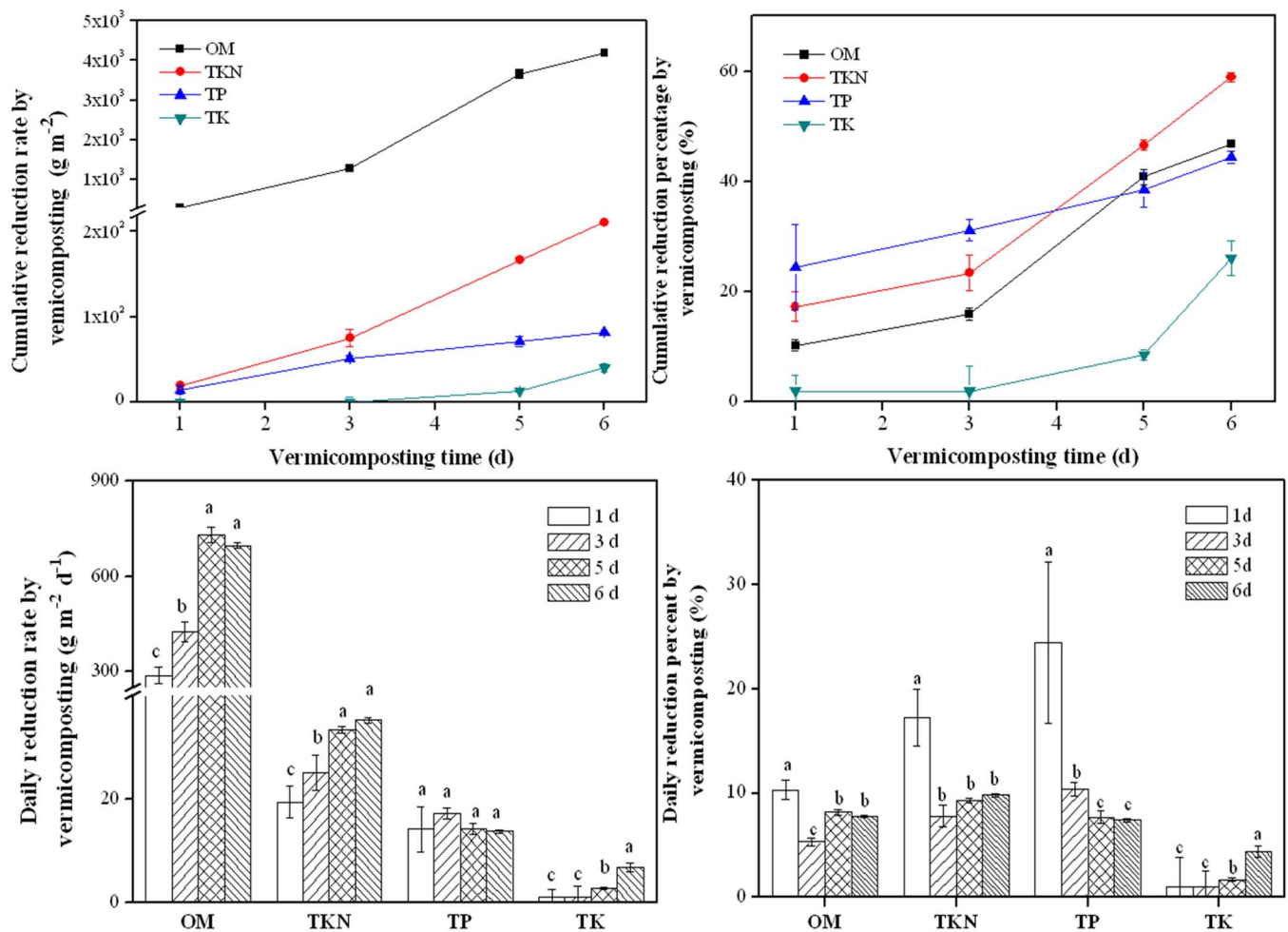


Figure 3 | Cumulative and daily reduction rates or percentages of organic matter (OM), total Kjeldahl nitrogen (TKN), total phosphorus (TP), and total potassium (TK) for vermicompost after 1 (T-1), 3 (T-3), 5 (T-5), and 6 days (T-6) during swine manure larvae vermicomposting.

with antibiotic attenuation. Raw manure has abundant OM, TKN, and active biological features and contained relative high concentrations of all nine antibiotics. Importantly, it was decomposed with a synchronous antibiotic reduction after vermicomposting using this process (Table S1). Results from this study revealed that the amount of manure reduction was correlated with the removal of antibiotics (Fig. 5), and a remarkable reduction of manure moisture (Fig. 2 and Fig. S1) produced rapid growth and metabolism of aerobic microorganisms^{23–25} due to oxygen enrichment of the vermicompost

material. This may be beneficial for antibiotic attenuation and suggests that vermicompost may be a safe approach for use in agriculture. It is noteworthy that QLs had a relatively lower rate of reduction than the other two classes of antibiotics during vermicomposting (Table 1, Fig. 1). There are two possible explanations for this observation. First, the low values of octanol-water partition coefficients ($\log K_{ow} = 0.61–1.48$) and high values of sorption coefficients ($K_d = 690–1270$, at 28°C) suggest that the adsorption of quinolones from sludge (e.g., electrostatic interaction) is much stronger than

Table 2 | Enzymatic activities ($\text{mmol h}^{-1} \text{g}^{-1}$ dry weight basis) of raw manure over time (C-0, C-3, C-6) and vermicomposts after 1 (T-1), 3 (T-3), 5 (T-5), and 6 days (T-6) during swine manure vermicomposting. Four hydrolytic enzymes involved in carbon, nitrogen and phosphorus cycling were investigated in this study, including β -1,4-glucosidase (β G), cellobiohydrolase (CBH), β -1,4-N-acetylglucosaminidase (NAG), and acid phosphatase (AP)

Treatment	β G		CBH		NAG		AP	
	Value	Ratio*	Value	Ratio	Value	Ratio	Value	Ratio
C-0	294 ± 23bc	—	633 ± 57b	—	534 ± 93b	—	34.8 ± 0.7c	—
C-3	312 ± 48bc	0.06	607 ± 35b	± 0.04	558 ± 55b	0.04	40.4 ± 3.5c	0.16
C-6	388 ± 26b	0.32	628 ± 41b	−0.01	512 ± 32b	−0.04	38.6 ± 9.6c	0.11
T-1	251 ± 11c	−0.15	758 ± 61a	0.20	722 ± 49a	0.35	65.5 ± 6.5a	0.86
T-3	377 ± 35b	0.28	749 ± 6a	0.18	653 ± 24ab	0.22	51.2 ± 1.3b	0.47
T-5	525 ± 40a	0.79	641 ± 26ab	0.01	545 ± 13b	0.02	47.9 ± 2.5b	0.38
T-6	369 ± 1b	0.19	535 ± 29c	−0.15	207 ± 28c	−0.61	25.9 ± 0.1d	−0.26

*The response ratio (Ratio) was calculated as: the difference of [mean of enzymatic activity in manure/vermicompost on the i-th day (d) minus mean of enzymatic activity in fresh manure on day 0 (i.e., C-0)] divided by the mean of enzymatic activity in fresh manure on day 0 (i.e., C-0).

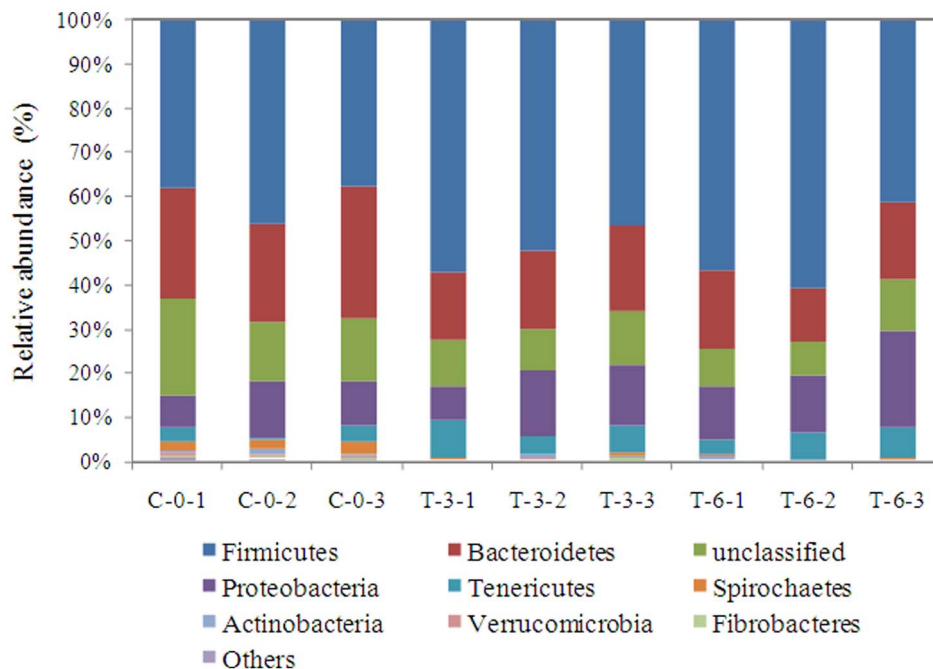


Figure 4 | The distribution of sequences in the dominant bacterial phyla from raw swine manure (C-0) and vermicompost after 1 (T-1), 3 (T-3), 5 (T-5), and 6 days (T-6). Relative abundances are based on the proportional frequencies of those DNA sequences that could be classified at the phylum level. The percent values are presented within the respective sections in the columns.

hydrophobic interactions^{21,26}. Thus, manure solids and/or vermicompost would be a temporary ‘refuge’ for OL persistence. Second, product analyses have shown that oxidation by MnO₂ results in dealkylation and hydroxylation at the piperazine moiety of QLs, with the quinolone ring remaining essentially intact²⁷. This low susceptibility to oxidation could easily maintain active QLs under the anaerobic-prevalent conditions during the process of larvae manure vermicomposting. Therefore, the environmental risk of QLs should be considered over other antibiotics when vermicompost is recycled as a biofertilizer.

The dynamic nature of manure/vermicompost enzymes acts as a medium to control the synchronous reduction of both manure waste and antibiotics, although a direct linkage between enzyme activities and manure antibiotic concentrations was not observed (Fig. 5). The intensities of βG and CBH dynamics have been previously linked to the rate of organic C biodegradation and the maturity of the waste composting process^{24,25}, since the product of CBH is the substrate for

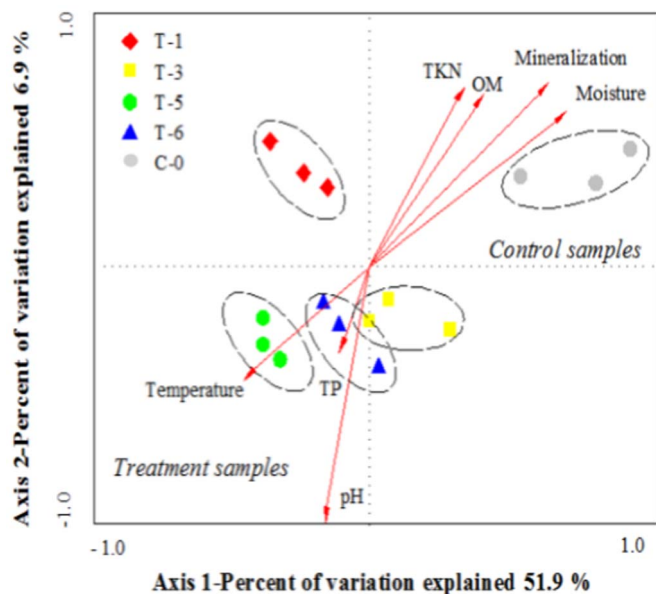


Figure 5 | The relationship between antibiotic attenuation and physico-biochemical variables by redundancy analysis (RDA).

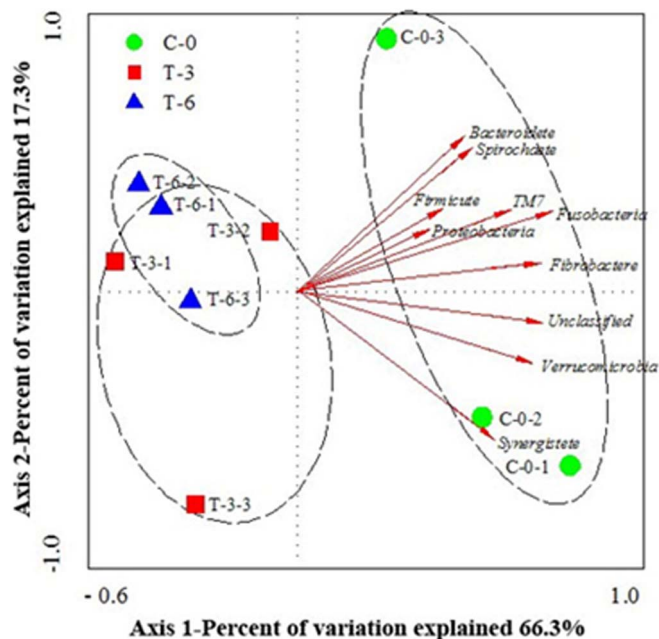


Figure 6 | The relationship between antibiotic attenuation and dominant bacterial phyla from raw manure (C-0) and vermicompost after 1 (T-1), 3 (T-3), 5 (T-5), and 6 days (T-6) during swine manure larvae vermicomposting.



the β G catalytic reaction²⁸. This “seesaw”-type co-existence of β G and CBH dynamics from day 1 to day 5 (Table 2) created a synergistic mechanism for the rapid biodegradation of organic C during vermicomposting (Fig. 3). The simultaneous drop in these two enzymes at the end of the vermicomposting process (at day 6) suggested an exhaustion of active organic C pools, leaving the steady and recalcitrant C in vermicompost. Organic N and P were rapidly biodegraded by larvae-microorganism complexes during the first few days of vermicomposting (Fig. 3) with enhanced NAG and AP activities (Table 2), suggesting a nutrient need for microbial metabolism in the vermireactor. The joint enzymatic reactions sped up the consumption of organic matter, accompanied by plentiful nutrients for microbial metabolism, which maintained the exposure of antibiotics to microbial attack. Previous studies have indicated that several antibiotics are hydrolytically susceptible to chemical bonds whose integrity is central to biological activity, such as esters and amides, and involves the oxidation–reduction processes due to the formation of an inactive compound^{29,30}. Not surprisingly, all of the enzymes detected in our study are involved in the reactions required to hydrolyze structural glucans, amino acids, and phosphates²⁸, which have evolved to target and cleave these vulnerable bonds³¹. Enzymatic catalysis increases the opportunity for antibiotics to lose their ‘harbor’. Therefore, the force of enzymatic activities could contribute to the positive impacts of OM, TKN, respiration intensity, and moisture to antibiotics (Fig. 5), representing a synchronous mechanism for waste reduction and antibiotic attenuation (Table 1, Fig. 3).

Firmicutes and *Proteobacteria* were the predominant bacterial phyla produced in the vermireactor (Fig. 4, $p < 0.05$), although the total bacterial population decreased during the process (Fig. S3). There was also a remarkable suppression of microbial respiration (Fig. 2d). Similarly, a study using an earthworm vermireactor fed by agroindustrial lignocellulose wastes confirmed a remarkable decline in bacteria abundance⁸. The decrease in the abundance of microorganisms could be due to two mechanisms: 1) rapid manure reductions (e.g., moisture, OM and TKN, in Fig. 2 & 3 and Fig. S2) and alterations in physical-chemical features of biomass (e.g., pH, temperature, and enzymatic activities, in Fig. 2 and Table 2) that occurred during vermicomposting generated an unfavorable environment for anaerobic-prevailing microorganisms as well as microbes that cannot compete with others for the limited nutrients^{8,32}; and 2) Pathogens cannot survive the gut fluids during the passage of waste through the gut (e.g., earthworms) and are eliminated and/or competitively displaced by the proliferation of other microorganisms^{32,33}. The enhanced proportions of these two phyla demonstrate an abundance of such bacteria to regulate hydrolytic enzyme activities for organic breakdown^{34–36}. During the bioconversion process, these serial reactions of microorganism-enzyme-waste directly impacted the degradation of manure/vermicompost antibiotics (Fig. 6). *Bacteroidetes* exhibited an intimate linkage with antibiotic concentration (Fig. 6), which was largely responsible for the degradation of high molecular weight organic matter, such as proteins and carbohydrates³⁷. During the later stages of vermicomposting, microbial mineralization severely decreased (Fig. 2d) and the bacterial population declined with the absence of three detected phyla (Fig. 4 and Fig. S3). The reduction in *Bacteroidetes* confirmed the presence of numerous carbohydrate-active enzymes covering a large spectrum of substrates³⁷, which promoted a highly decomposed stage for manure waste and had a positive effect on antibiotic attenuation (Fig. 6). Antibiotics, however, are among the most important groups of pharmaceuticals and chemotherapeutic agents that inhibit or terminate the growth of microorganisms, such as CFC³⁸. The manure, with an abundant bacterial population and concentration of antibiotics (Fig. S3 and Table S1), was stabilized and matured under the vermi-culture created by larvae-microorganism complexes⁵. The structural transition of the bacterial community

(Fig. 4 and Fig. S3) was conducive for accelerating the biodegradation process of antibiotic attenuation (Fig. 6). A similar study reported that the bacterial degradation for doxycycline (DOC) and OTC resulted in a large decrease in more than 50% of the initial antibiotic concentration³⁹, suggesting that the level of bacterial degradation was sufficient to affect the dynamics of antibiotic residue. The response of the bacterial population in manure/vermicompost impacts the waste decomposition process through enzyme catalysis by introducing a readily biodegradable substance, allowing for the monitoring of effects on manure waste, and offering an underlying mechanism for antibiotic attenuation at the genetic level.

To the best of our knowledge, this is the first report on the antibiotic removal efficiency using a full-scale, semi-continuous feeding vermireactor. Based on the analysis above, there are two feasible measures that can be taken for technological improvement: 1) an enhancement of feeding manure from day-1 to day-3 for efficiency promotion, since the maximum percentage of daily reduction for antibiotic residues mainly appeared at T-1 and T-3 (Table 1 and Fig. 1); and 2) increasing and maintaining high temperature in the vermireactor, particularly during the winter season and the early stages of vermicomposting, because the higher temperature seems beneficial for antibiotic attenuation (Fig. 5). Although an alkaline condition accelerates the degradation of antibiotics (Fig. 5) and abiotic transformation²³, a higher pH inside the vermireactor would cause a greater release of ammonia from the manure residue^{14,40}. Thus, technical caution is needed when commercial alkaline is added to manure during larvae vermicomposting for purposes of improving antibiotic attenuation. Enzyme catalysis and microflora composition may contribute to the removal of swine manure antibiotics via larvae vermireactors. In addition, manure microbiota might be modified, since the gut microbiome of most organisms is dominated by *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*^{41,42}, representing more than 1000 different molecular species or phylotypes⁴³. Further research should focus on the functional potential of certain bacteria through combined phylogenetic, taxonomic, and metagenomic analyses⁴⁴ in order to better understand the underlying antibiotic degradation mechanisms. Moreover, the use of veterinary antibiotics may result in the selection of antibiotic-resistant bacteria, which could challenge microbial populations and impact the structure and activity of environmental microbiota^{14,40}. The same resistance genes found in clinical settings are currently disseminated among pristine ecosystems without any record of antibiotic contamination⁴⁰ due to the high diversity and prevalence of mobile plasmids carrying antibiotic genes in the parent substance¹⁴. These may result in a potential hazard on environmental safety and human health¹⁸. Due to relatively low biodegradation, the persistence of QLs in animal waste (Fig. 1) could cause serious genetic selection of QL-related resistance genes, since the relative abundance of these genes and the concentrations of QLs were shown to be significantly correlated⁴⁵. Based on these conclusions, further investigation of whether this full-scale vermireactor can impede resistant bacterial pathogens and plasmid-mediated genes is warranted.

Methods

Design and operation of full-scale larvae vermireactor. In September 2008, a full-scale swine manure vermicomposting operation using housefly (*Musca domestica*) larvae was installed by the HangZhou TianYuan Agriculture Development Co., Ltd. (30°49′47.02″N, 120°39′22.12″E) located in XiaoShan District, HangZhou, Southeast China. The firm currently possesses 35,000 m² of greenhouse-assisted larvae vermireactors with a maximum daily treatment capacity of 105 tons per day of fresh raw manure. Raw swine manure (with no solid-liquid separation) was collected from a neighboring swine farm raising approximately 155,000 finishing hogs annually.

The design of the full-scale swine manure vermicomposting facility has been described in detail in previously reports^{1,5} and can be found in the supplemental material S1. Briefly, four main processing units are involved in the vermicomposting process: 1) seed-fly breeding and fly oviposition, 2) larvae vermicomposting, 3) larvae-vermicompost separation, and 4) seed stocking and eclosion (Fig. S4 and Fig. S5). The greenhouse-assisted larvae vermireactors were operated with a semi-continuous feeding mode: 11.0 kg raw manure (moisture content 78–84%, Fig. S2) per m²



was laid as the base feed in each vermireactor at the beginning of vermicomposting; as increasing amounts of manure were consumed by the housefly larvae, new layers of raw manure were added sequentially at the rate of 5.56, 15.6, 15.6, and 5.56 kg m⁻² (wet weight) on day-1, day-2, day-3 and day-4, respectively; no additional raw manure was added on day-5 and day-6. The manure in the vermireactor reached an average depth of 8–10 cm at the completion of composting. Manure solids (with moisture of 45%) after solids-liquid separator (sieve opening: 2.5 mm) were added at rate of 6.5 kg m⁻² (wet weight) into and mixed with the stack of vermicomposting on day-5, in order to improve the permeability and accelerate the biological dehydration before larvae-vermicompost separation on day 6.

Manure and vermicompost sampling. A batch of raw swine manure was piled under ambient conditions with no larvae addition after 0, 3, and 6 days (Control: C-0, C-3, C-6), and vermicompost (before raw manure feeding) during vermicomposting after 1, 3, 5, and 6 days (Treatment, T-1, T-3, T-5, and T-6, respectively) were collected in triplicates in May 2013. Vermicompost samples (1.0 kg) were taken from four randomly chosen sampling sub-sections (diameter 6 cm) in each vermireactor with a cylindrical stainless-steel container. Temperature and pH in middle layer of manure were recorded for both Control and vermicomposting treatment. After uniform mixed, sub-samples were collected for analysis. All samples were immediately placed in a portable cooler, and transported to the laboratory in Zhejiang University within 2 h and stored at -20°C for no more than 8 h before further processing. The samples thereafter were freeze-dried (Labconco, Kansas City, MO) and homogenized by sieving through a 0.15 mm mesh before analysis.

Extraction and analysis of antibiotics. Nine antibiotics, belonging to three classes, frequently detected in environmental samples^{11,12} were investigated in this study: 1) TCs: tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and doxycycline (DOC); 2) SAs: sulfadiazine (SDZ); 3) QLs: norfloxacin (NFC), ofloxacin (OFC), ciprofloxacin (CFC) and enrofloxacin (EFC). The concentrations of antibiotics in manure/vermicompost samples were determined by liquid chromatography-tandem mass spectrometry with the isotope-labeled internal standard method as previously described¹¹. Briefly, an aliquot (0.2 g) of each sample was spiked with the surrogate standards and internal standards (100.0 µg kg⁻¹), followed with 20 mL of extraction solvent (EDTA-sodium phosphate buffer with acetonitrile: Mg(NO₃)₂·NH₃·H₂O, v/v, 3:1). The extraction mixtures were placed in the dark overnight and centrifuged at 5000 rotations per minute (rpm) for 10 min after rigorous mixing for 30 min at 200 rpm at 25 ± 1°C in the dark and ultrasonication for 15 min. The supernatant containing antibiotics was collected and the same extraction protocol was repeated two more times for the residue. The supernatants from the three extractions were combined, filtered through a 0.7 mm glass microfibre filter (GF/F, Whatman) and diluted to 500 mL with ultrapure water to maintain the organic solvent content <5% in the solution. Then the antibiotics were extracted using ultrasonic-assisted extraction followed by solid phase extraction clean-up with hydrophilic-lipophilic balance cartridges. The antibiotics analytes were separated and detected by liquid chromatography-electrospray ionization tandem mass spectrometry and quantified by the isotope-labeled internal standard method. Mass spectral acquisition was done in the positive ion mode by applying multiple reactions monitoring two fragmentation transitions per analyte to provide a high degree of sensitivity and specificity. The calibration range used for all the antibiotics was 5.0–300 µg L⁻¹.

Chemical analysis. Chemical analysis of manure/vermicompost samples were performed at HangZhou Center for Inspection and Testing for Quality and Safety for Agricultural and Genetically Modified Products, Ministry of Agriculture, PR China (<http://www.nybzjzh.com/index.asp>) following standard methods for the quantification of nutrients and organic matter. Briefly, the moisture content of the swine manure and larvae casts was determined after drying at 105°C for 24 h. Organic matter (OM) was determined the weight difference before and after heating at 550°C for 4 h. Total Kjeldahl nitrogen (TKN) was determined as alkali NH₃ liberation following nitrogen conversion to NH₄⁺-N by digestion with concentrated H₂SO₄ and HClO₄. Total phosphorus (TP) was analyzed using the molybdenum antimony colorimetric method after digestion in concentrated H₂SO₄ and HClO₄. Total potassium (TK) was determined after digesting 0.3 g dry sample in a di-acid mixture (HNO₃:HClO₄ = 4:1, v/v) by flame photometry.

Biochemical measurements. Four enzymes related to the biodegradation of carbon (C), nitrogen (N), and phosphorus (P) were selected as indicators of microbial activities: β-1,4-glucosidase (βG), cellobiohydrolase (CBH), β-1,4-N-acetylglucosaminidase (NAG), and acid phosphatase (AP). Fluorometric enzyme assays for βG, CBH, NAG and AP were conducted for manure samples using methylumbelliferyl-linked (MUB) substrates as previously described^{28,46}. Briefly, sample suspensions were prepared by homogenizing 1 g (wet weight) of sample with 125 mL of 50 mmol L⁻¹ sodium acetate buffer (pH 6.5) using a vortex shaker for 1 min. Assays were conducted in 96-well blank fluorescent plates (Corning Inc., costar 3603, USA). The substrates for the four enzymes were 4-MUB-β-D-glucoside, 4-MUB-β-D-cellobioside, 4-MUB-N-acetyl-β-D-glucosaminide and 4-MUB-phosphate, for βG, CBH, NAG and AP, following a previously described protocol⁴⁶. The micro-plates were covered and incubated in the dark at 20°C for 4 h. At the end of the assay, 10 µL of 1.0 mol L⁻¹ NaOH were added to each well to stop the reaction and induce fluorescence. Fluorescence was measured 1 min after NaOH addition to reduce signal noise²⁸. Fluorescence was measured with a Bio-Tek Synergy HT

microplate reader (Bio-Tek Inc., Winooski, VT, USA) with 365 nm excitation and 460 nm emission filters. Enzyme activities were calculated and expressed as nmol h⁻¹ g⁻¹.

Microbial respiration rate was estimated as the rate of CO₂ evolution according to a previously described method²⁴. Samples (10 g wet weight equivalent) were incubated in airtight containers at 24°C in the dark. Evolved CO₂ was captured with a 1.0 mol L⁻¹ NaOH solution, which was titrated with HCl to the phenolphthalein endpoint.

454 pyrosequencing of bacterial communities in manure and vermicompost.

Community DNA was extracted from 0.2 g of samples using the Ezup genomic DNA extraction kit (Sangon Biotech, Shanghai, China) according to the manufacturer's protocol. Extracted DNA was quantified using a Nanodrop spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington). An aliquot (50 ng) of purified DNA from each sample were used as the template for amplification of the V3–V6 regions of bacterial 16S rRNAs using the following primer set⁴⁷: 341F (5'-CCTACGGGAGGCGAGCAG-3) with the Roche 454 'A' pyrosequencing adapter and a unique 7 bp barcode sequence, and 1073R (5'-ACGAGCTGACGACARCCATG-3) with the Roche 454 'B' sequencing adapter at the 5-end of each primer respectively. Polymerase chain reaction (PCR) were carried out in 20 µL reaction mixtures containing 4 µL 5 × FastPfu Buffer, 2 µL 2.5 mmol L⁻¹ dNTPs, 0.8 µL 5 µmol L⁻¹ Forward Primer, 0.8 µL 5 µmol L⁻¹ Reverse Primer, 0.4 µL FastPfu Polymerase, 10 ng Template DNA and add ddH₂O to 20 µL. Each reaction mix was amplified under the following conditions: 2 minutes at 95°C, 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s; with a final extension at 72°C for 5 min. PCR products were pooled and purified by Agarose Gel DNA purification kit (Sangon Biotech, Shanghai, China). The PCR products from each sample were combined in equimolar ratio in a single tube and run on a Roche FLX 454 sequencer (Roche Diagnostics Corporation, Branford, CT, USA). Sequences have been deposited into the NCBI short-reads archive database (Accession number: SRA00128). Procedures of sequence analysis were provided in the supplemental materials (S2).

Statistical analysis. All assays were conducted in triplicates. The cumulative reduction rate, daily reduction rate, cumulative reduction percentage and daily reduction percentage of the vermicompost antibiotics, OM, TKN, TP or TK were estimated by these formulas:

$$\text{Cumulative reduction rate (mg m}^{-2}\text{)} = \frac{(\sum C_{Ri} \times M_{Ri}) - C_{Vi} \times M_{Vi}}{S} \quad (1)$$

$$\begin{aligned} \text{Daily reduction rate (mg m}^{-2}\text{d}^{-1}\text{)} &= \frac{\text{Cumulative reduction rate}}{T_i} \\ &= \frac{(\sum C_{Ri} \times M_{Ri}) - C_{Vi} \times M_{Vi}}{S \times T_i} \end{aligned} \quad (2)$$

$$\text{Cumulative reduction percentage} = \frac{(\sum C_{Ri} \times M_{Ri}) - C_{Vi} \times M_{Vi}}{\sum C_{Ri} \times M_{Ri}} \times 100\% \quad (3)$$

$$\begin{aligned} \text{Daily reduction percentage} &= \frac{\text{Cumulative reduction percentage}}{T_i} \\ &= \frac{(\sum C_{Ri} \times M_{Ri}) - C_{Vi} \times M_{Vi}}{(\sum C_{Ri} \times M_{Ri}) \times T_i} \times 100\% \end{aligned} \quad (4)$$

where C_{Ri} , C_{Vi} are the antibiotic, OM, TKN, TP or TK concentrations of raw manure and vermicompost on the i -th day, respectively (Table S1, Fig. S1). M_{Ri} , M_{Vi} are manure inputs and mass of vermicompost left on the i -th day. S is the basal area of one vermireactor. T_i is vermicomposting time on the i -th day.

Analysis of variance (ANOVA) was performed with SPSS Statistical software version 17.0, using the S-N-K test for multiple comparison. The relationships among taxonomic diversity in the microbial communities and antibiotic attenuation and environmental variables were evaluated with redundancy analysis (RDA) using CANOCO 4.5, with phylum/class data entered as supplementary variables.

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Author contributions

Z.J.Z. and X.H.X. conceived and designed the study. J.G.S., H.W., C.F.Z. and M.L. conducted field sampling and biochemical & molecular sequencing. L.H.W., F.P. and H.Y.L. performed antibiotics analysis and data processing. Q.H. and H.W. operated statistic analysis. All authors assisted with drafting the manuscript. Z.J.Z., J.G.S., H.W. and X.H.X. revised the manuscript.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

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