Effects of different biochars on antibiotic resistance genes during swine manure thermophilic composting

Jian Wang[1,2], Bin Sui[2,3], Yujun Shen[1,2], Haibo Meng[1,2*], Lixin Zhao[2,3], Haibin Zhou[1,2], Ran Li[1,2], Jingtao Ding[1,2], Hongsheng Cheng[1,2], Xi Zhang[1,2]

(*1. Institute of Energy and Environmental Protection, Chinese Academy of Agricultural Engineering Planning and Design, Beijing 100125, China*; 2. *Key Laboratory of Technologies and Models for Cyclic Utilization from Agricultural Resources, Ministry of Agriculture and Rural Affairs of the People's Republic of China, Beijing 100125, China*; 3. *Chinese Academy of Agricultural Engineering Planning and Design, Beijing 100125, China*)

Abstract: Elimination of antibiotic resistance genes (ARGs) in animal manure from concentrated animal feeding operations by thermophilic composting has drawn increasing attention. This study investigated the effects of sawdust biochar, corn stover biochar and peanut hull biochar with three spiked levels on ARGs in swine manure during thermophilic composting. Thirteen ARGs corresponding to four classes of antibiotics (tetracyclines, sulfonamides, macrolides and quinolones) were determined in the composting piles. Results indicated that the ten tested composting groups became fully mature after 30-day thermophilic composting process. *tet*M, *tet*O and *erm*B were reduced in all tested groups; *tet*C and *tet*G, *tet*X, *sul*1, *sul*2, *erm*F, *qnr*D and *aac(6')-Ib* were mostly reduced under low level biochar addition but increased under higher level biochar addition; *gyr*A increased under medium biochar addition and reduced in other groups; *oqx*B remained comparatively stable throughout the composting process. The addition levels of spiked biochar are more important than types of spiked biochar on the removal of ARGs in the composting pile. The average removal rates of ARGs in the control group, low, medium and high level biochar addition groups were 0.24 logs, 0.52-0.72 logs, –0.52-0.18 logs and –0.19-0.21 logs, respectively. In summary, low level biochar addition could enhance the elimination of studied ARGs in swine manure during the composting process, while medium level biochar addition to the composting piles would increase the risk of ARGs' propagation.

Keywords: biochar, antibiotic resistance genes, thermophilic composting, swine manure
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1 Introduction

Antibiotics are widely and heavily used in Chinese livestock and poultry industries. In 2013, up to 84 000 tons of veterinary antibiotics were used in China, and this amount contributed to 52% of the total antibiotic usage in China[1]. The lack of efficient treatment of animal manure resulted in high residual level of antibiotics. The detected residual concentrations of antibiotics in animal manure could reach to g/kg grade[2]. As a result, the residual antibiotics in animal manure for growth promotion and disease control has led to the dissemination of antibiotic resistance genes (ARGs)[3-5].

Composting, which can transform animal manure into organic fertilizer, is a commonly and widely adopted technology in China. Existing studies have shown that composting can effectively remove the antibiotics in broiler[6], pig[7,8] and cow[9,10] manure. Besides, the behaviors of ARGs during manure composting process were also studied, but the results were somewhat distinct. Although many studies reported that composting could reduce ARG levels in animal manure[11-13], some studies showed that ARG levels were not sufficiently reduced after composting[14-16]. The reduction of ARGs may depend on temperature[11], aerobic condition[12] and moisture content[16] of the composting pile.

Biochar is a kind of solid product obtained from pyrolysis of biomass residues in the absence or deficiency of oxygen. As an additive in composting, biochar can reduce the contamination potentials of composting product, such as reducing the emissions of ammonia[17-19], odors and greenhouse gases[20,21], as well as deactivating the heavy metals[22]. The addition of biochar could also promote the removal of ARGs. Li et al.[23] found that addition of bamboo charcoal during chicken manure composting could reduce the relative abundances of most studied ARGs by 0.85-1.15 logs. Cui et al.[24] reposted that different biochar types (such as rice straw and mushroom biochar) and manure types (such

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Biographies: **Jian Wang,** Engineer, research interests: cyclic utilization from agricultural resources, Email: wangjian@caaepd.org.cn; **Bin Sui,** Professor, research interests: agricultural and rural construction and cyclic utilization from agricultural resources, Email: suibin@caaepd.org.cn; **Yujun Shen,** Senior Engineer, research interests: cyclic utilization from agricultural resources, Email: shenyujun@caaepd.org.cn; **Lixin Zhao,** Professor, research interests: energy resource utilization of agricultural wastes, Email: zhaolixin@caaepd.org.cn; **Haibin Zhou,** Senior Engineer, research interests: treatment and utilization of agricultural wastes, Email: zhouhaibin@caaepd.org.cn; **Ran Li,** Assistant Engineer, research interests: fertilizer utilization of agricultural wastes, Email: liran@caaepd.org.cn; **Jingtao Ding,** Senior Engineer, research interests: cyclic utilization from agricultural resources, Email: dingjingtao@caaepd.org.cn; **Hongsheng Cheng,** Senior Engineer, research interests: fertilizer utilization of agricultural wastes, Email: chenghongsheng@caaepd.org.cn; **Xi Zhang,** Engineer, research interests: fertilizer utilization of agricultural wastes, Email: zhangxi@caaepd.org.cn.

***Corresponding author: Haibo Meng,** Professor, research interests: cyclic utilization from agricultural resources. Chinese Academy of Agricultural Engineering Planning and Design, No. 41, Maizidian Street, Chaoyang District, Beijing 100125, China. Tel: +86 10 59197288, Fax: +86 10 59197276, Email: menghaibo@caaepd.org.cn.

as duck and swine manure) influenced the behaviors of ARGs in different ways. Sawdust, corn stover and peanut hull were abundant biomass resources for biochar production in China. However, the effects of biochars produced by the three biomass types on ARGs during manure composting still remain largely unknown.

Therefore, a series of simulated swine manure thermophilic composting tests with addition of three kinds of biochars were carried out to investigate the behaviors of tetracycline resistance genes (TRGs), sulfonamide resistance genes (SRGs), fluoroquinolone resistance genes (QRGs) and macrolide resistance genes (MRGs). The aims of this study were to investigate the effects of different types and levels of biochars addition on ARGs during swine manure composting and to evaluate the most appropriate spiked proportion of biochar addition for eliminating ARGs in the composting piles.

2 Materials and methods

2.1 Composting materials

The composting materials comprised a mixture of swine manure, corn stover (approximate 1 cm), and three types of biochars (sawdust, corn stover and peanut hull biochar). Swine manure was collected from a swine farm in Shunyi District, Beijing. Corn stover was collected from the surrounding farmland in Daxing District, Beijing. Biochars were made from sawdust, corn stover and peanut hull, respectively, at a pyrolysis temperature of 600 ℃. The physicochemical properties of the materials are shown in Supporting information.

2.2 Composting operation

Ten simulated composting groups were prepared. For each composting group, 24 kg of fresh manure was mixed with 3 kg of corn stover using a blender to adjust the final C/N ratio to 30 and the final moisture content to 65%. Biochars were spiked to the composting mixture at levels of low (6%), medium (12%) and high (24%) on a dry weight basis. Ten composting groups including: Control (swine manure + corn stover), SB6 (sawdust biochar 6% + swine manure + corn stover), SB12 (sawdust biochar 12% + swine manure + corn stover), SB24 (sawdust biochar 24% + swine manure + corn stover), CB6 (corn stover biochar 6% + swine manure + corn stover), CB12 (corn stover biochar 12% + swine manure + corn stover), CB24 (corn stover biochar 24% + swine manure + corn stover), PB6 (peanut hull biochar 6% + swine manure + corn stover), PB12 (peanut hull biochar 12% + swine manure + corn stover), PB24 (peanut hull biochar 24% + swine manure + corn stover). Afterward, each tested group of composting mixture was put into a bench-scale composting bioreactor[25]. The aeration rate of each group is 0.1 $m^3/(min \cdot m^3)$ using an intermittent aeration mode with 5 min of aeration followed by 30 min without aeration over the whole composting process, which lasted for 30 days. Composite samples were collected by mixing three subsamples that collected from the bottom, middle and top layers of each pile on Day 0 and 30.

2.3 DNA extraction and quantitative PCR (qPCR)

Total DNA was extracted from 0.2 g of each composite sample by using a FastDNA SPIN kit for soil (MP-bio, USA). The concentration and quality of the extracted DNA were determined by spectrophotometric analysis (Genequant 1300, GE Healthcare, USA) and agarose gel electrophoresis, respectively. The extraction and analysis of DNA were performed in triplicate for each sample.

Thirteen ARGs, including five TRGs (i.e., *tet*C, *tet*G, *tet*X, *tet*M, *tet*O), two SRGs (i.e., *sul*1 and *sul*2), four QRGs (i.e., *gyr*A, *qnr*D, *aac(6')-Ib* and *oqx*B), two MRGs (i.e., *erm*B and *erm*F), as well as the 16S rRNA genes, were quantified using the q-PCR method. Primers used for each gene in this study were according to our former study[16] and other studies[26-30]. The temperature program consisted of initial denaturation at 95 ℃ for 30 s, followed by 40 cycles of 15 s at 95 ℃, 20 s at different annealing temperatures and extension at 72°C for 30 s, and finished with melt-curve analysis from 60 ℃ to 95 ℃. Ten-fold serial dilutions (10^8 to 1 gene copy number) of the plasmid DNA were performed to establish the standard curve. All the R^2 of standard curve were more than 0.99. As the absolute abundance of ARGs was severely impacted by the total extracted biomass of each sample, the relative abundance of ARGs (copy number of ARG/copy number of 16S rDNA) was used in this study.

2.4 Statistical analysis

All statistical analyses were performed using SPSS version 19.0, and the paired samples *t*-test based on the *p*-value was used to assess the homogeneity of variance with significance levels of 5% ($p<0.05$).

3 Results and discussion

3.1 Evaluation of composting maturity

According to the US EPA standard regarding the time and temperature requirements for biosolids in-vessel composting, which is "55 ℃ for at least 3 d"[31], it indicates that the cumulative high temperature (i.e., cumulative temperature above 55 ℃) is crucial for the maturity of composting piles. The cumulative high temperature of ten tested composting groups ranged from 287.4 ℃ d to 868.5 ℃ d and exceeded the US EPA standard (165 ℃ d) (Figure 1). Compared with CK, the addition of peanut hull biochar (PB6, PB12 and PB24) increased the value of cumulative high temperature of composting piles, while the addition of other two tested biochars showed no significant effect on cumulative high temperature. In addition, the germination indexes (GI) of ten composting groups after 30 d of composing were 83.1%-119.5%, indicating the maturity of composting piles.

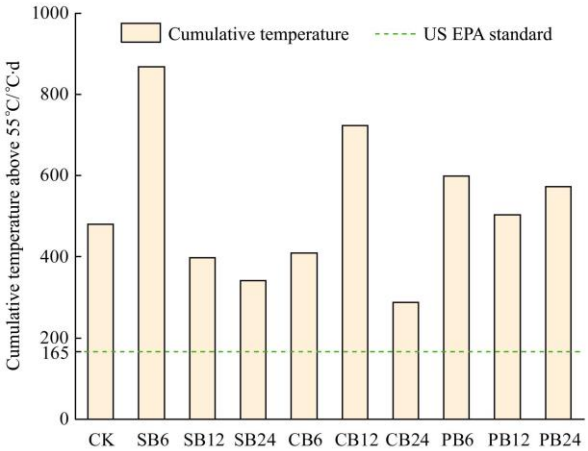

Figure 1 Cumulative temperature of composting piles

3.2 Behaviors of TRGs

Ribosomal protection (RP), efflux pump (EFP) and enzymatic inactivation (EI) proteins were three main mechanisms of TRGs[32,33]. In this study, two EFP TRGs (*tet*C and *tet*G), two RP TRGs (*tet*M and *tet*O) and one EI TRG (*tet*X) were determined in the tested composting groups. The variation of their relative abundances was presented in Figure 2. The log-transformed removals of ARGs were calculated based on their relative

abundances in the raw and composted piles. During the composting process, the relative abundance of TRGs in the tested composting groups varied from 1.25×10^{-4}-1.42×10^{-1} in the beginning to 2.75×10^{-6}-1.42×10^{-2} in the end, and the log-transformed removals of TRGs were –1.93-2.35 logs. RP TRGs were removed in all tested groups by 0.61-2.35 logs. Higher addition of sawdust biochar could enhance the removal of RP TRGs (p<0.05). Comparatively, EFP TRGs and EI TRG were mostly removed by –1.85-1.06 logs in SB6, CB6 and PB6, but increased in other tested groups by –0.39-1.93 logs. It was reported that the hosts of RP TRGs were mainly anaerobic bacteria and prone to diminish during aerobic composting, while the hosts of EFP and EI TRGs were most commonly aerobic and facultative Gram-negative bacteria[16,32]. Thus, RP TRGs were more likely to be eliminated than EFP and EI TRGs after aerobic composting. The addition of biochar could increase the duration of the thermophilic phase; on the other hand, with the specialty of structure and nutrients, biochar is a suitable habitat and carbon source for microbial communities[34]. Hence, low level addition (6%) of biochar may enhance the thermophilic effect of composting piles to kill some heat-liable bacteria then foster the ARGs elimination, while high level addition (12% and 24%) of biochar to composting piles could better promote the microbial proliferation and lead to the propagation of ARGs they carried.

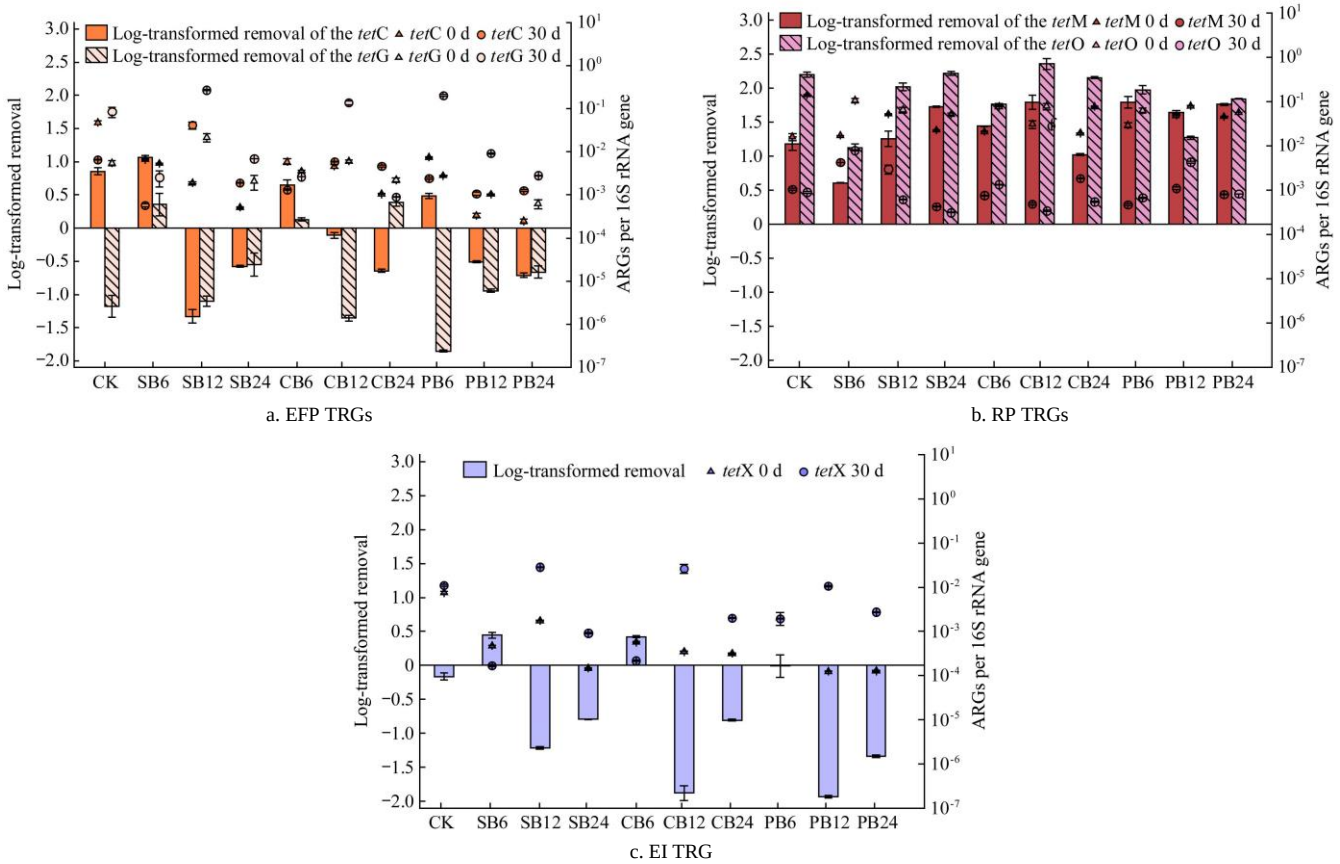

Figure 2 Log-transformed removal and relative abundances of EFP TRGs, RP TRGs and EI TRGs in the composting piles

3.3 Behaviors of SRGs and MRGs

The mutant dihydropteroate synthase (DHPS) genes are the main sulfonamide resistance gene type. *sul*1 and *sul*2 were most commonly mutant DHPS genes[35]. Figure 3a presented the variation of SRGs in the tested composting groups. The relative abundance of tested SRGs varied from 1.16×10^{-4}-8.61×10^{-2} at the beginning of composting to 1.53×10^{-4}-7.79×10^{-2} in the end, and the log-transformed removals of SRGs were –1.54-0.42 logs. Results in this study showed that the addition of biochars led to an overall increment of *sul*1 by –0.02-1.08 logs; similarly, *sul*2 were reduced by 0.15-0.42 logs only under low level biochar addition (6%) but increased by 0.65-1.54 logs under high level biochar addition (12% and 24%). As *sul*1 and *sul*2 are often associated with mobile gene elements such as plasmids and integrons, their dissemination could be very efficient[35], indicating that they are more recalcitrant to the microbial communities evolution during composting than other chromosome-borne ARGs. Thus, as the addition of biochars could enhance the microbial activities in the tested composting groups, the relative abundance of SRGs would also be increased along with this process.

The *erm* genes were important MRGs related to macrolide-lincosamide-streptogramin B antibiotic multi-resistance phenotype[36], and often related to transposons. The variation of *erm*B and *erm*F in the tested composting groups were shown in Figure 3b. During the composting process, the relative abundance of them varied from 9.40×10^{-4}-8.28×10^{-2} in the beginning to 2.00×10^{-5}-3.65×10^{-3} in the end, and their log-transformed removals were –1.34-2.57 logs. Similar to the behaviors of SRGs, *erm*B and *erm*F were reduced by 0.5-2.41 logs under low level biochar addition (6%). However, when the dose of biochars increased, *erm*B was reduced by 1.20-2.57 logs while *erm*F was increased by –0.02-1.34 logs, manifesting opposite variation patterns. *erm*B is commonly related to Tn1545 and Tn916 conjugative transposons[37], and *erm*F is often related to Tn5030 conjugative transposon[38]. A previous study found that *erm*F was highly transferable among clinical anaerobic and aerobic bacteria[39]. The different gene location might influence their behaviors.

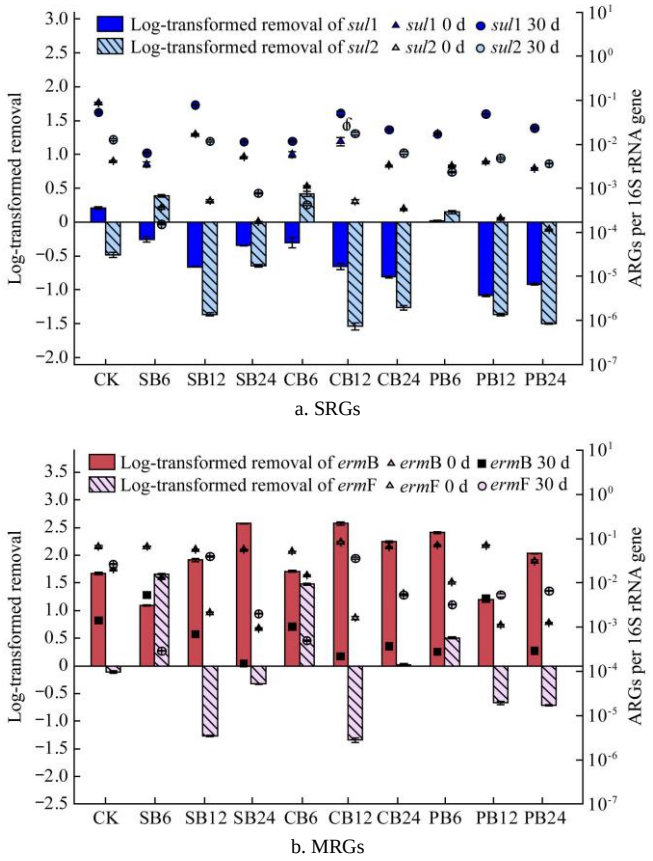

Figure 3 Log-transformed removal and relative abundances of SRGs and MRGs in the composting piles

3.4 Behaviors of the quinolone resistance genes

Target-mediated (TM) QRG (i.e. *gyr*A) and plasmid-mediated (PM) QRGs (i.e. *oqx*B, *qnr*D and *aac(6')-Ib*) were determined in this study. Figure 4 showed the profiles of QRGs in the tested composting groups. Compared with other ARGs, the variations of different QRGs showed distinct patterns. The relative abundance of *gyr*A ranged from 5.71×10^{-4}-2.77×10^{-2} at the beginning of composting to 3.41×10^{-4}-1.61×10^{-2} when the composting was finished (Figure 4a). *gyr*A decreased by 0.29-0.69 logs in SB6, SB24, CB6, CB24 and PB6, and increased by 0.42-1.33 logs in CK, SB12, CB12, PB12 and PB24. *oqx*B was relatively stable in all tested group with the relative abundance of 1.79×10^{-3}-8.41×10^{-3}, and increased by 0.11-0.43 logs in the nine biochar-added groups (Figure 4b). In comparison, *qnr*D and *aac(6')-Ib* decreased by 0.46-1.33 logs in under low level biochar addition (6%), and mostly increased by 0.12-2.48 logs under high level biochar addition (12% and 24%) (Figure 4c). The dramatic differences of QRGs' variations could be attributed to many reasons. It seemed that medium dosage of biochars (12%) could foster the propagation of *gyr*A. A possible explanation may be that *gyr*A is usually chromosome-borne ARG and proliferates depending on its host bacteria, moderate dosage of biochars (12%) might facilitate the reproduction of its host. *oqx*B was reported to be highly abundant in swine wastewater from Beijing, China[40]. In addition, the plasmids carrying *oqx*B were proved to be of high mobility, which could promote their maintenance and transmissibility[41]. Thus, this might contribute to the stability of *oqx*B during the composting. *qnr*D and *aac(6')-Ib* are also located on plasmids but their variations were different from that of *oqx*B, probably because that *oqx*B is located on larger plasmids which may limit their dissemination between different species or general of bacteria[27], while unlike *oqx*B, *qnr*D and *aac(6')-Ib* are often located on smaller plasmids that could propagate by the active microbial activities during composting[42,43].

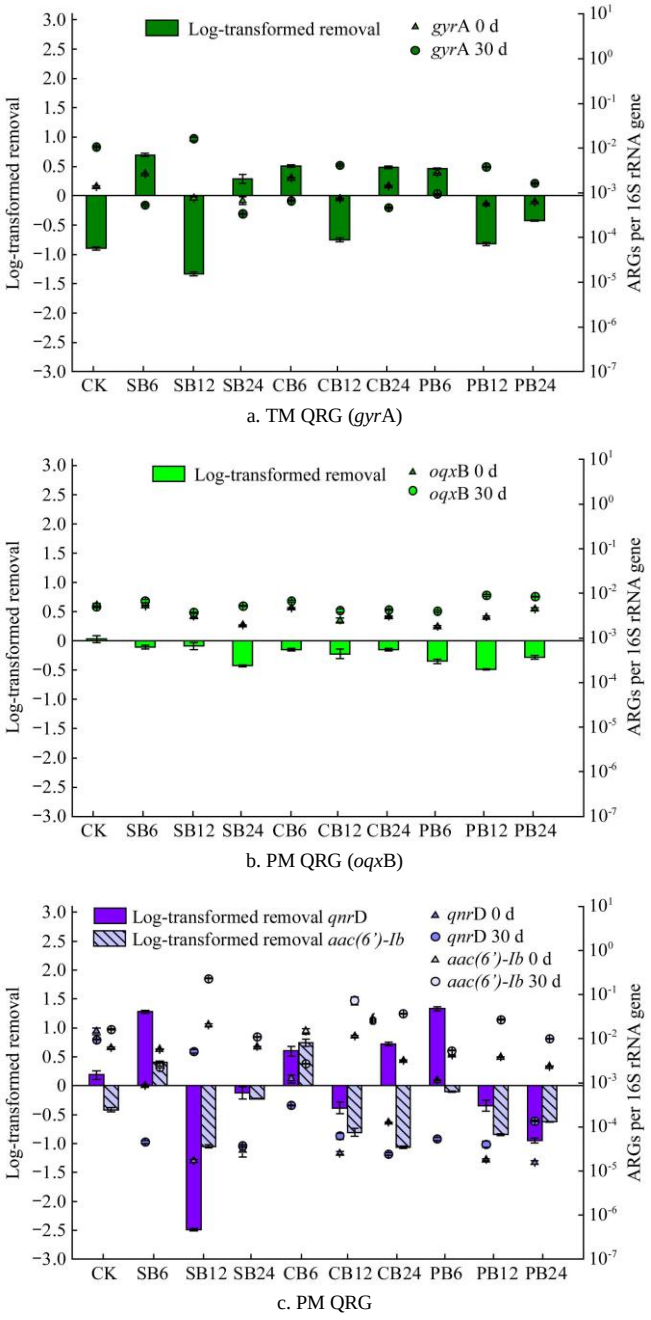

Figure 4 Log-transformed removal and relative abundances of QRGs *gyr*A, *oqx*B, *qnr*D and *aac(6')-Ib* in the composting piles

3.5 Evaluation of ARGs' removal by different biochars

The overall log-transformed removals of thirteen ARGs were presented in Figure 5. The average values of log-transformed removal in the ten tested groups were –0.52-0.72 logs and there was no significant difference among different biochar types. It is clear from the figure that the average removal rates of ARGs in groups of low level biochar addition (SB6, CB6 and PB6) were the highest compared with other groups, which were 0.67, 0.72 and 0.52 logs, respectively, and the rate of CB6 was higher than that of CK (0.24 logs) at significant level ($p<0.05$). Groups of medium level biochar addition (SB12, CB12 and PB12) achieved the lowest average removal rates of ARGs (–0.52-0.18 logs), of which the rates of SB12 and PB12 were lower than that of CK at extremely significant level ($p<0.01$). Groups of high level biochar addition

(SB24, CB24 and PB24) achieved the medium average removal rates of ARGs (–0.19-0.21 logs). Few studies had evaluated the effects of various biochars under different spiked levels on the removal of ARGs during thermophilic composting. Cui et al.[44] evaluated the addition of rice straw biochar and mushroom biochar on the behaviors of ARGs during chicken manure composting and found that the addition of the two biochars achieved opposite effects on the ARGs, which may be due to their different influences on the bacterial communities. Duan et al.[45] also reported that the bacterial community succession caused by biochar addition was the main mechanism that affected the variations of ARGs and *int*I1 in soil amendment. Our results showed that the spiked levels of biochars affected the removal of ARGs more significantly than the types of biochars. As the proliferation of ARGs were mainly depending on the reproduction of their host bacteria, their different behaviors in the test groups may also due to the changes of bacterial communities affected by different types and levels of biochars, which needs further study.

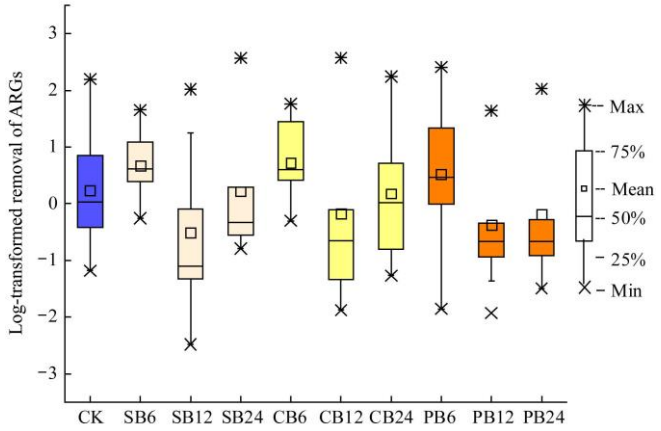

Figure 5 Distribution of log-transformed removals of tested ARGs in the composting piles

4 Conclusions

In this study, a series of simulated swine manure thermophilic composting tests with addition of three kinds and three spiked levels of biochars were carried out to investigate the variations of four classes of ARGs. Based on the experimental results, the following conclusions can be drawn:

Ten tested composting groups became fully mature after 30 days. RP TRGs (*tet*M and *tet*O) were reduced in all tested groups. EFP TRGs (*tet*C and *tet*G), EI TRG (*tet*X), SRGs (*sul*1 and *sul*2) and two PM QRGs (*qnr*D and *aac(6')-Ib*) were mostly reduced under low level biochar addition (6%) but increased under higher level biochar addition (12% and 24%). MRGs (*erm*B and *erm*F) were reduced under low level biochar addition but behaved the opposite variation patterns under higher level biochar addition. TM QRG (*gyr*A) was promoted under medium biochar addition and reduced in other groups. PM QRG (*oqx*B) remained comparatively stable throughout the composting. Spiked levels of biochars are more important than types of biochars added to the composting piles on the removal of ARGs. Low level biochar addition could enhance the removal of ARGs, while medium level biochar addition would foster the propagation of ARGs.

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