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牛粪堆肥系统环境因子对抗性基因的影响

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摘要: 本研究利用实时定量PCR技术检测牛粪60 d好氧堆肥过程中典型四环素类抗性基因(*tetQ*、*tetW*、*tetM*、*tetG*、*tetA*和*tetX*)和大环内酯类抗性基因(*erm*35、*erm*36、*ermB*、*ermF*、*ermT*和*ermX*)的数量变化规律,系统研究了温度、含水率、挥发性有机质(VS)含量、碳氮比(C/N)、pH值、氧化还原电位(ORP)等堆肥系统环境因子和细菌数量(TCB)对这两类典型抗生素抗性基因的影响. 结果表明,堆肥升温阶段牛粪中*tetQ*、*tetW*、*tetG*和*tetX*数量分别增加1.7、0.3、84.8和4.5倍,而*tetM*丰度降低了83.1%;堆肥腐熟阶段牛粪中*tetG*和*tetX*数量分别增加23.8和11.5倍,而*tetW*、*tetQ*和*tetM*数量均降低90%. 整个牛粪堆肥过程中*erm*35、*erm*36、*ermB*、*ermF*、*ermT*和*ermX*丰度分别增加2.1、430.4、0.6、11.5、2.1和49.1倍. RDA分析表明,牛粪堆肥过程中对四环素与大环内酯类抗性基因数量影响较大的3个环境因子分别为: ORP(54.8%)、温度(34%)和VS(11.3%),其中ORP与*ermX*和*ermB*、温度与*tetQ*和*ermF*以及VS与*tetW*数量均呈明显地正相关.

关键词:牛粪; 抗性基因; 堆肥; 四环素; 大环内酯; 环境因子
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Effects of Environmental Factors on Tetracycline and Macrolides Resistance Genes in Cattle Manure Composting Systems

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Abstract: The effects of environmental factors and bacterial numbers on typical tetracycline resistance genes (*tetQ*, *tetW*, *tetM*, *tetG*, *tetA*, and *tetX*) and macrolide resistance genes (*erm*35, *erm*36, *ermB*, *ermF*, *ermT*, and *ermX*) during cow manure composting were investigated and quantified via real-time quantitative PCR. The results suggested that the abundance of *tetQ*, *tetW*, *tetG*, and *tetX* increased by 1.7, 0.3, 84.8, and 4.5 times respectively, whereas *tetM* decreased by 83.1% during the heating stages of cow manure composting. The abundance of *tetG* and *tetX* increased by 23.8 and 11.5 times respectively, whereas *tetW*, *tetQ*, and *tetM* decreased by 90% during the maturity stage. However, the macrolide resistance genes (*erm*35, *erm*36, *ermB*, *ermF*, *ermT*, and *ermX*) increased by 2.1, 430.4, 0.6, 11.5, 2.1, and 49.1 times, respectively. Redundancy analysis showed that ORP, temperature, and VS were crucial factors that could explain the variations of 54.8%, 34%, and 11.3%, respectively, in ARGs. ORP, temperature, and VS also had the greatest positive correlation with *ermX*, *ermB*, *tetQ*, *ermF*, and *tetW*, in that order.

Key words: cow manure; resistance genes; composting; tetracyclines; macrolides; environmental factors

近年来,由于抗生素在养殖业中的广泛使用,导致了大量耐药菌和抗生素抗性基因(antibiotic resistance genes, ARGs)在自然环境中广泛出现. 抗生素抗性基因作为一种新型环境污染物,它可通过质粒、可移动遗传元件和整合子等介质进行不同微生物之间的传播和扩散[1,2],会对人类身体健康产生巨大伤害. 目前四环素类和大环内酯类的抗生素被广泛用于畜禽养殖业中,我国畜禽粪便产量巨大,也是环境中这两类抗生素抗性基因的主要来源. 如果畜禽粪便处理不当,会直接造成抗生素抗性基因的传播[3~5],在畜禽类粪便及周边废水、沼气残渣、废水处理系统和土壤等样品中均会检测到四环素类和大环内酯类抗性基因[6~8]. 因此畜禽粪便如何处理受到广泛关注.

高温好氧堆肥可将有机固体废物转化为稳定的有机肥料,可以有效除去粪便中的抗生素和致病微生物,是目前国内外畜禽类粪便处理及资源化的重要途径[9~11]. 好氧堆肥过程中温度、湿度、有机质和pH等环境因子会显著变化,从而导致微生物种群结构演替[12,13],这可能会潜在影响畜禽粪便中抗生素抗性基因的丰度和归趋[8,14]. 勾长龙等[15]检测猪粪高温堆肥后*tetA*相对丰度增加,*tetG*、*tetM*、*tetQ*和*tetW*的相对丰度均下降. Li等[16]检测鸡粪堆肥后*tetG*、*tetW*、*tetX*、*ermB*、*ermF*和*ermX*的相对丰度均降低. 但它们在牛粪中的变化情况还不清楚. 因此,本研究检测了牛粪好氧堆肥不同阶段典型四环素类抗性基因(*tetQ*、*tetW*、*tetM*、*tetG*、*tetA*和*tetX*)和大环内酯类抗性基因(*erm*35、*erm*36、*ermB*、

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ermF、*ermT* 和 *ermX*)的数量变化规律,探究堆肥过程中温度、含水率、有机质含量(VS)、碳氮比、pH 值和 ORP 变化对两类典型抗性基因分布的影响. 堆肥是目前国内外畜禽粪便处理的主要技术,研究堆肥处理过程中环境因子与抗性基因的相关关系对于控制抗生素抗性基因污染具有重要意义.

1 材料与方法

新鲜牛粪取自哈尔滨郊区某畜牧场. 将新鲜牛粪与木屑(桦树)按 2.5∶1(质量比)的比例混合后放于有机玻璃堆反应器中,进行为期 60 d 的好氧发酵处理. 堆肥反应器通风速率为 2.5 $L\cdot min^{-1}$,每隔 10 d 对堆肥物料进行翻堆. 从反应堆顶部、中部和底部随机采取样品,将其混合成代表性样品. 检测 60 d 堆肥不同时期样品含水率、pH 值、氧化还原电位(ORP)、挥发性有机质(VS)含量、碳氮比(C/N)等理化参数的变化规律.

将堆肥样品置于 105℃ 的烘箱中干燥至恒重,根据初始重量和干燥后质量测定牛粪含水率. 再将牛粪放于 550℃ 的马弗炉中 2 h,残差法测定样品 VS 含量. 将样品研磨并筛选(0.075 mm 筛网),用元素分析仪检测总碳、总氮和 C/N. 将 5 g 样品加入 50 mL ddH_2O,振荡 2 h 后用 pH/ORP 计测定 pH 和 ORP,每项参数 3 次重复检测以减少误差. 每日利用温度计测量并记录堆肥物料温度和环境温度.

利用 MoBio 公司的超净土壤 DNA 提取试剂盒提取样品 DNA,按照说明书进行操作. DNA 用 1% 琼脂糖凝胶检测. 在 ABI 7500 system 中对 6 个四环素类的抗性基因(*tetM*、*tetG*、*tetQ*、*tetW*、*tetA* 和 *tetX*)、6 个大环内酯类抗性基因(*erm35*、*erm36*、*ermB*、*ermF*、*ermT* 和 *ermX*)和细菌总数进行定量分析,引物序列参照表 1[17,18]. qPCR 反应体系(20 μL):10 μL SYBR® Premix Ex Taq Ⅱ(2 ×),2 μL DNA 模板,0.4 μL ROX Reference Dye Ⅱ(50 ×),0.8 μL 正反向引物(10 $\mu mol\cdot L^{-1}$),6 μL ddH_2O. 反应条件:95℃ 15 min,95℃ 10 s,退火温度 30 s,72℃ 1 min,循环 40 次. 试验数据采用 Microsoft Excel 和 Origin Pro 9.0 软件进行整理与分析,并利用 CANOCO 5.0 软件进行冗余分析,分析堆肥过程中抗性基因与环境因子的相关性.

表 1 PCR 引物序列

Table 1 PCR Primer Sequence

抗性基因	引物序列	
tetG	F: AGAATCTGCTGTTTGCCAGTG	R: CGGAGTGTCAATGATATTGCA
tetM	F: ACAGAAAGCTTATTATATAA	R: TGGCGTGTCTATGATGTTCA
tetQ	F: CGCCTCAGAAGTAAGTTCATACACTAAG	R: TCGTTCATGCGGATATTATCAGAAT
tetW	F: GAGAGCCTGCTATATGCCAGC	R: GGGCGTATCCACAATGTTAAC
tetA	F: GCGCGATCTGGTTCACTCG	R: AGTCGACAGYRGCGCCGGC
tetX	F: AAATTTGTTACCGACACGGAAGTT	R: CATAGCTGAAAAAATCCAGGACAGTT
*erm*35	F: TTGAAAACGATGTTGCATTAAGTCA	R: TCTATAATCACAACTAACCACTTGAACGT
*erm*36	F: GGCGGACCGACTTGCAT	R: TCTGCGTTGACGACGGTTAC
ermB	F: GCTTTATTTCAGGAGGCGGAAT	R: TTTTAAATGCCACAGCACAGAATC
ermF	F: CAGCTTTGGTTGAACATTTACGAA	R: AAATTCCTAAAATCACAACCGACAA
ermT	F: CATATA AATGAAATTTTGAG	R: ACGATTTGTATTTAGCAACC
ermX	F: GCTCAGTGGTCCCCATGGT	R: ATCCCCCCGTCAACGTTT
16S rRNA	F: CCTACGGGAGGCAGCAG	R: ATTACCGCGGCTGCTGG

2 结果与讨论

2.1 堆肥过程中环境因子的变化特征

好氧堆肥不同时期牛粪理化参数变化特征详见表 2. 堆肥过程中易降解有机质被微生物逐渐利用[19],牛粪中 VS 含量由最初 83.7% 降至堆肥第 60 d 的 63.1%,经过 60 d 堆肥处理后牛粪中碳含量由 36.7% 降至 22.3%. 温度是影响堆肥系统中微生物活性的重要参数(如图 1). 堆肥初期有机物分解释放热量导致堆体温度上升,并在堆肥第 3 d 达到 54℃,从而使堆肥系统进入高温阶段. 随着牛粪中易降解有机含量逐渐降低,物料温度逐渐下降,并在第 30 d 左右接近环境温度,这标志着好氧堆肥一次发酵结束腐熟阶段开始. 堆肥系统高温和通风条件促进物料中的水分挥发,堆肥处理后牛粪含水率由初始 79.7% 降至 38.2%.

堆肥初期牛粪中蛋白质水解释放氨氮,牛粪的 pH 值由 6.5 上升至第 30 d 的最大值 8.8. 有机质的矿化导致 C/N 由最初 14.6 上升至第 30 d 的 16.5. 高温和水分蒸发造成堆肥中氨气挥发,使堆肥后期牛粪的 pH 值逐渐降低. 堆肥后期温度降低堆体中微生物数量增加,消耗堆体中有机物导致 C/N 降低. 牛粪 EC 值从 20.6 $mS\cdot cm^{-1}$ 降至 2.1 $mS\cdot cm^{-1}$,这可能是由于牛粪中水溶性化合物含量

的减少以及腐殖质的生成[20,21]. 除此之外,牛粪 ORP 值从 -109 mV 显著上升至 162 mV,这可能是由于堆肥过程中微生物对还原性化合物的生物氧化作用[22].

表 2　牛粪堆肥过程中理化参数的变化

Table 2　Changes in physicochemical parameters during cow manure composting

理化参数	堆肥时间			
	新鲜牛粪	第 10 d	第 30 d	第 60 d
pH	6.5 ±0.2	8.4 ±0.1	8.8 ±0.2	8.1 ±0.1
EC/mS·cm^{-1}	20.6 ±0.3	14.8 ±0.3	8.7 ±0.2	2.1 ±0.2
ORP/mV	-109 ±0.6	42 ±3.9	135 ±1.01	162 ±2.6
VS/%	83.7 ±0.3	76.6 ±0.3	68.5 ±0.1	63.1 ±0.1
N/%	2.5 ±0.1	1.9 ±0.1	1.8 ±0.1	2.1 ±0.1
C/%	36.8 ±0.2	30.1 ±0.3	30.1 ±0.1	22.3 ±0.4
C/N	14.6 ±0.3	16.2 ±0.2	16.5 ±0.4	10.9 ±0.3
含水率/%	79.7 ±0.1	71.6 ±0.1	62.0 ±0.6	38.2 ±0.3

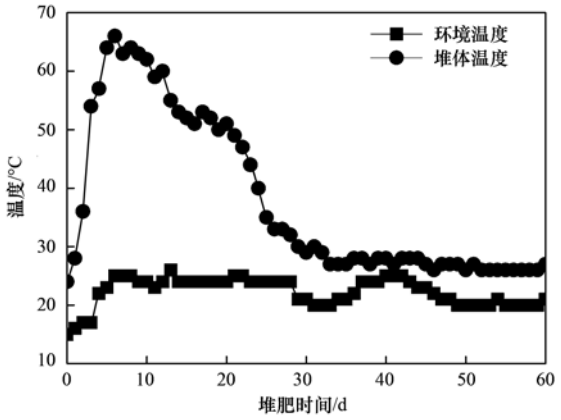


图 1　堆肥过程中物料温度和环境温度的变化情况

Fig. 1　Changes in material temperature and environmental temperature during cow manure composting

2.2　四环素类抗性基因的变化特征

本研究利用 qPCR 技术检测了堆肥过程中 6 种典型四环素抗性基因的数量变化情况(见图 2),其中包括 3 个编码核糖体保护蛋白抗性基因(*tetM*、*tetQ* 和 *tetW*)、2 个编码外排泵蛋白基因(*tetA* 和 *tetG*)和 1 个酶修饰抗性基因(*tetX*). *tetM* 含量在新鲜牛粪中最高 1.0×10^4 copies·g^{-1},并随着好氧堆肥处理时间逐渐减少. 堆肥高温阶段牛粪中 *tetQ* 和 *tetW* 数量达到最高值,分别为 1.4×10^4 和 1.1×10^5 copies·g^{-1},这与 Su 等[23]发现 ARGs 富集在堆肥嗜热阶段的结果一致. *tetQ* 和 *tetW* 数量在腐熟阶段后期下降至 4.9×10^2 和 7.8×10^3 copies·g^{-1}. Guo 等[24]也发现猪粪堆肥后 ARGs 丰度会降低 8.1% ~ 96.7%,其中 *tetW* 减少 90% 以上. 与之相反,牛粪中 *tetG* 含量在堆肥第 30 d 达到最高 4.7×10^4 copies·g^{-1}, *tetX* 数量由新鲜牛粪的 5.0×10^3 copies·g^{-1}逐渐上升至堆肥第 60 d 的 6.3×10^4 copies·g^{-1},而 *tetA* 在整个堆肥过程中未检测到.

Wang 等[25]发现堆肥处理会有效减少污泥中四环素类抗性基因的含量. Yu 等[26]也发现在猪粪堆肥中 *tetM*、*tetO*、*tetQ*、*tetS*、*tetT*、*tetW*、*tetA* 和 *tetG* 的丰度均会有所降低. 尽管本研究牛粪堆肥过程中 *tetM*、*tetQ* 和 *tetW* 含量同样有所降低,但是与新鲜牛粪相比,堆肥产品中 *tetG* 和 *tetX* 基因数量是明显增加的,这与 Zhang 等[27]在污泥堆肥中 *tetX* 和 *tetG* 拷贝数增加的结果一致. 堆肥原料的理化性质、微生物种群结构和堆肥系统环境因子均可能会导致好氧发酵过程中抗性基因变化趋势的差异.

2.3　大环内酯类抗性基因的变化特征

牛粪堆肥过程中大环内酯类抗性基因数量变化情况如图 3 所示. 一次发酵过程中抗性基因 *ermB*、*ermX*、*ermT* 和 *erm*35 含量均显著增加,并在堆肥第 30 d 达到最高值,分别为 2.9×10^3、5.8×10^3、1.5×10^3 和 9.6×10^4 copies·g^{-1},这一结果与 Guo 等[24]发现在猪粪堆肥中 *ermX* 丰度减少量超过 90% 明显相反. 抗性基因 *erm36* 丰度一次发酵过程中数量很低,但是其在腐熟阶段有所升高. 另外,牛粪中 *ermF* 含量在高温阶段(第 10 d)达到最大值(8.6×10^5 copies·g^{-1})后逐渐降低. 本研究结果表明堆肥产品中 6 种大环内酯类抗性基因数量与新鲜牛粪相比均有所增加,可见好氧堆肥并不能去除所有的 ARGs,而且导致部分大环内酯类抗性基因含量有所增加. 郑宁国[28]发现在猪粪堆肥过程中 *ermB* 和 *ermC* 丰度降低而 *ermF* 丰度增加,相似污泥堆肥过程中 *ermB* 丰度降低而 *ermF* 丰度增加[29,30]. Liao 等[31]发现在污泥中 *ermB*、*ermT* 和 *ermX* 丰度降低而 *ermF* 丰度增加. 由此可见,牛粪、污泥和猪粪这 3 种底物在堆肥处理过程中 *ermB* 丰度变化特点并不完全相同.

2.4　抗性基因与细菌数量的相关性

细菌总数在堆体升温过程中显著升高,堆肥第 10 d 达到最大值 2.2×10^8 copies·g^{-1},在此之后堆肥样品中细菌总数开始逐渐降低[图 4(a)]. 由于堆肥系统中抗性基因数量与细菌数量密切相关,本

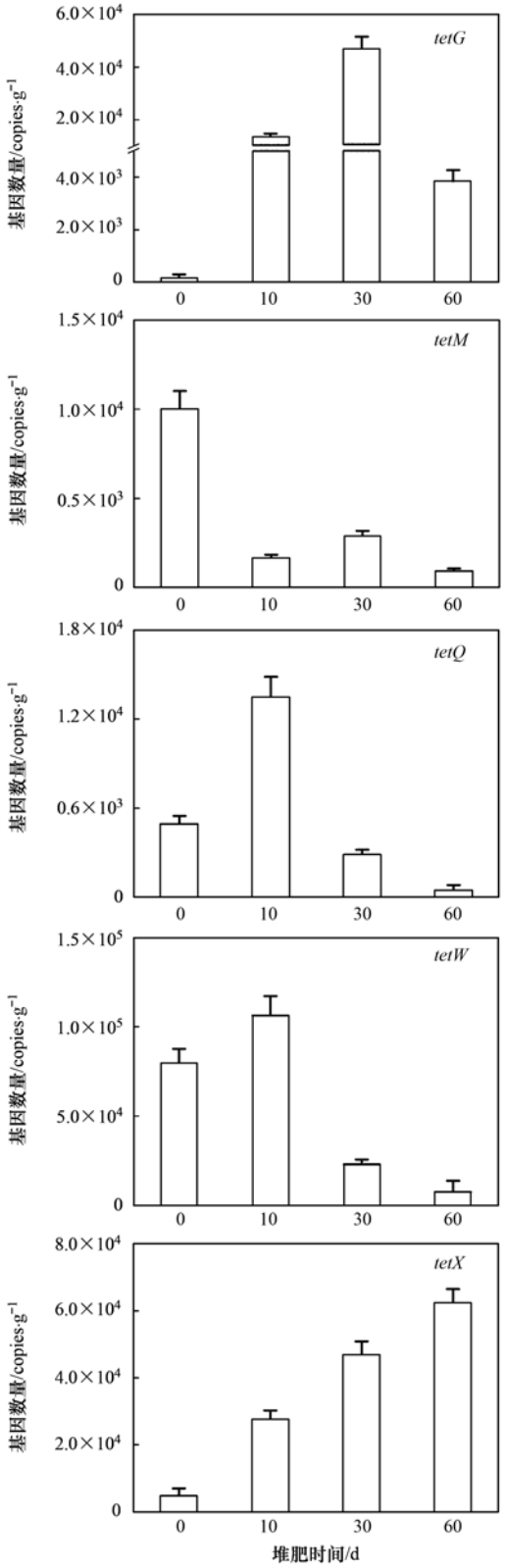


图 2 牛粪堆肥过程中四环素类抗性基因数量的变化

Fig. 2 Quantity changes in tetracycline resistance genes during cow manure composting

研究分别考察了堆肥过程中上述 6 种四环素类抗性基因和 6 种大环内酯类抗性基因与细菌总数相对丰度的变化规律[图 4(b)和 4(c)]. 在笔者检测的 6 种典型四环素类抗性基因中,新鲜牛粪中 *tetM* 和 *tetW* 相对丰度最高而 *tetG* 丰度最低. 尽管堆肥第 10 d *tetQ*、*tetW*、*tetG* 和 *tetX* 数量有所上升,但是全部 6 种四环素类抗性基因的相对丰度均有所下降,一方面堆肥高温环境对部分嗜温细菌具有较强的抑制作用,另一方面这些抗性基因可能具有相同的宿主细胞或是它们的宿主细胞间存在正相关[32,33]. 经过 60 d 堆肥处理后 *tetX* 相对丰度明显高于其它 5 种四环素类抗性基因,成为牛粪堆肥产品中最主要的四环素类抗性基因. 对比数据发现牛粪堆肥中 *tetM*、*tetQ* 和 *tetW* 减少幅度大于 *tetG*. Peng 等[34]发现鸡粪堆肥过程中 *tetM* 减少幅度大于 *tetG*. Yu 等[26]发现猪粪堆肥过程中 *tetM*、*tetQ* 和 *tetW* 均减少,而 *tetG* 没有明显变化. 编码核糖体保护蛋白基因(*tetM*、*tetQ* 和 *tetW*)的主要是革兰氏阳性菌,而编码外排泵蛋白基因(*tetA* 和 *tetG*)的主要是革兰氏阴性菌,革兰氏阳性菌比革兰氏阴性菌更容易去除,致使堆肥处理后 *tetM*、*tetQ* 和 *tetW* 减少幅度大于 *tetG*[7,34,35].

与四环素类抗性基因的变化规律相似,牛粪堆肥中 *erm35*、*erm36*、*ermT*、*ermB* 和 *ermX* 这 5 种大环内酯类抗性基因的相对丰度也在高温阶段达到最低,分别为 3.9×10^{-6}、0、4.8×10^{-7}、7.6×10^{-6} 和 3.8×10^{-6}. 这一研究结果表明尽管高温阶段抗性基因数量与细菌总数呈正相关,但这一时期大部分抗性基因的相对丰度却明显降低. 另外,整个堆肥过程中 *ermF* 的相对丰度明显高于其它 5 种大环内酯类抗性基因. *ermF* 的相对丰度在高温阶段最高(3.9×10^{-3})且随后变化不显著,成为牛粪堆肥产品中最主要的大环内酯类抗性基因. 韦蓓等[29]发现在污泥堆肥中 *ermB* 和 *ermF* 相对丰度在高温阶段降低而 *ermF* 相对丰度在冷却和腐熟阶段有所升高. Liao 等[31]发现在污泥堆肥中 *ermT*、*ermB* 和 *ermX* 在高温阶段降低而 *ermF* 相对丰度在冷却和腐熟阶段升高,本实验结果与其一致.

2.5 堆肥系统抗性基因与环境因子的相关性

本研究采用冗余分析法(RDA)评价堆肥系统温度、含水率、VS、C/N、pH 和 ORP 等环境因子以及细菌总数与上述 12 种典型抗生素抗性基因数量的相关关系(如图 5). 研究数据表明牛粪堆肥过程中对抗性基因分布规律影响最重要的 3 个环境因子分别为 ORP(54.8%)、温度(34%)和 VS(11.3%). 抗性基因 *tetW* 和 *tetM* 的丰度与挥发性有机质含量呈正相关,其中对 *tetW* 的影响最大. 抗性基因(*tetX*、*ermX* 和 *ermB*)的丰度与 ORP 呈正相关,但对 *ermX* 和 *ermB* 丰度影响程度最大. Wang 等[13]发现在污泥堆肥过程中微生物群落受 ORP、温度和含水率影响较大,而堆肥中微生物结构的变

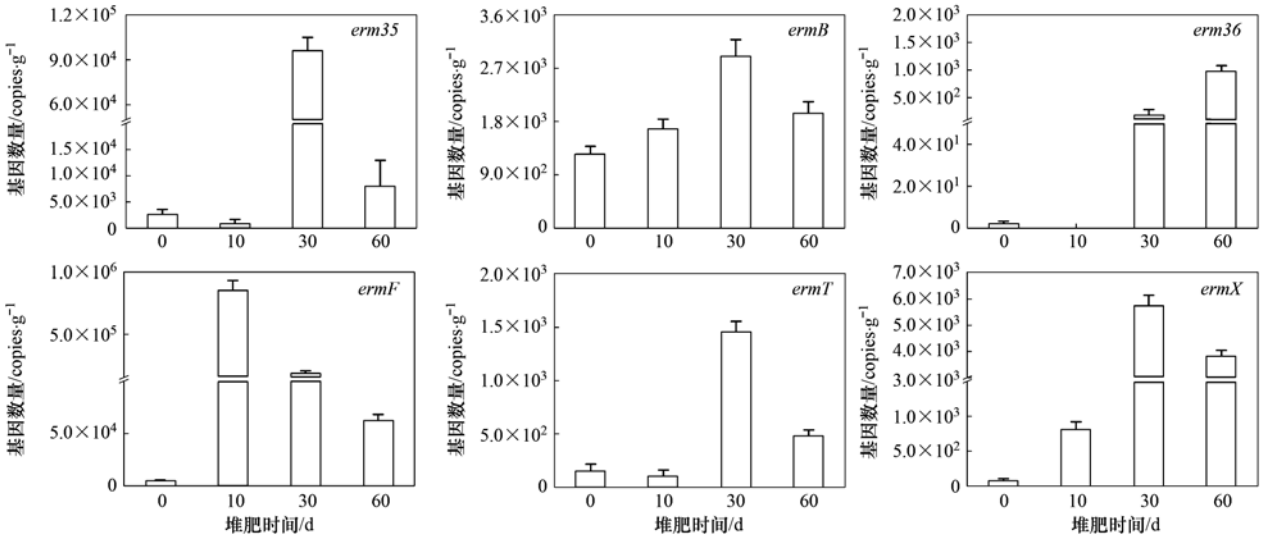
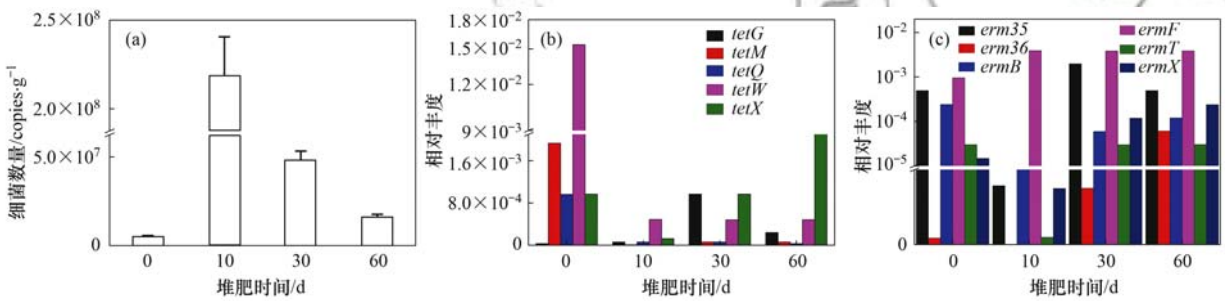


图 3　牛粪堆肥过程中大环内酯素类抗性基因数量的变化

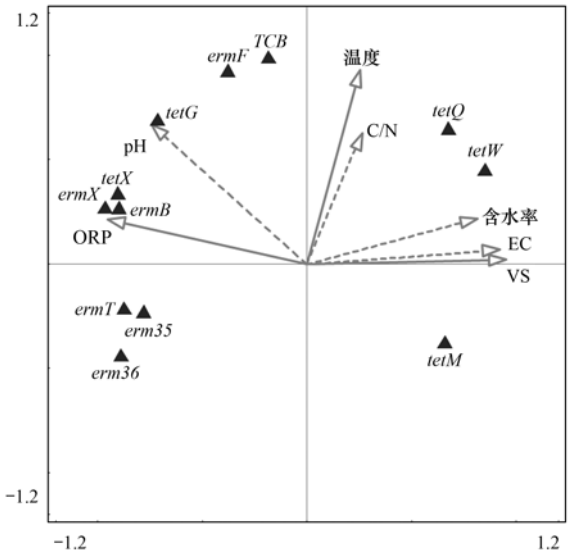
Fig. 3　Quantity changes in macrolide resistance genes during cow manure composting



(a)细菌数量;(b)四环素类抗性基因相对丰度;(c)大环内酯类抗性基因相对丰度

图 4　堆肥过程中细菌数量与抗性基因相对丰度的变化规律

Fig. 4　Relationship between resistance genes and bacterial quantities during cow manure composting



实虚箭头线:抗性基因分布变异影响是否重要的环境因子,
实线:重要因子,虚线:次要因子,三角形:抗性基因

图 5　抗性基因与环境因子的关系

Fig. 5　Relationship between resistance genes and environmental factors

化会显著影响 ARGs 分布. 抗性基因 *tetQ* 和 *ermF* 的丰度和细菌总数与温度呈正相关且有相同的变化趋势,其中温度对细菌总数影响程度最大. Li 等[16]发现添加竹炭的鸡粪堆肥过程中 ARGs 受到温度、C/N 和 pH 的影响较大. Zhang 等[36]发现不同表面活性剂处理鸡粪堆肥过程中 ARGs 受到温度、pH 和水溶性碳的影响较大. 堆肥过程中温度是影响微生物活性和分布的重要参数[37,38],是破坏和灭活耐药细菌和 ARGs 的关键变量[36].

3　结论

牛粪堆肥过程中 *tetM*、*tetQ* 和 *tetW* 数量显著减少,*tetG* 和 *tetX* 含量有所升高,而且与新鲜牛粪相比,经过 60 d 堆肥处理后 6 种大环内酯类抗性基因数量均有所增加. 在笔者检测的 12 种典型抗生素抗性基因中,牛粪堆肥产品中 *tetX* 和 *ermF* 含量最高. 好氧堆肥高温阶段牛粪中 *tetM*、*tetQ*、*tetW*、*tetX*、*erm35*、*erm36*、*ermB*、*ermT* 和 *ermX* 的相对丰度显著降低. 冗余分析表明温度、ORP 和 VS 分别

是影响牛粪堆肥系统中 *ermF*、*tetQ*、ermX、*ermB* 和 *tetW* 含量的主要环境因子.

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