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# Stockpiling versus Composting: Effectiveness in Reducing Antibiotic-Resistant Bacteria and Resistance Genes in Beef Cattle Manure

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**ABSTRACT** Manure storage methods can affect the concentration and prevalence of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in cattle manure prior to land application. The objective of this study was to compare stockpiling and composting with respect to their effectiveness in reducing ARB and ARGs in beef cattle manure in a field-scale study. Field experiments were conducted in different seasons with different bulking agents for composting. For both the winter-spring cycle and the summer-fall cycle, ARB concentrations declined below the limit of quantification rapidly in both composting piles and stockpiles; however, ARB prevalence was significantly greater in the composting piles than in the stockpiles. This was likely due to the introduction of ARB from bulking agents. There was no significant change in ARG concentrations between initial and final concentrations for either manure storage treatment during the winter-spring cycle, but a significant reduction of the ARGs *erm*(B), *tet*(O), and *tet*(Q) over time was observed for both the composting pile and stockpile during the summer-fall cycle. Results from this study suggest that (i) bulking agent may be an important source of ARB and ARGs for composting; (ii) during cold months, the heterogeneity of the temperature profile in composting piles could result in poor ARG reduction; and (iii) during warm months, both stockpiling and composting can be effective in reducing ARG abundance.

**IMPORTANCE** Proper treatment of manure is essential to reduce the spread of antibiotic resistance and protect human health. Stockpiling and composting are two manure storage methods which can reduce antibiotic-resistant bacteria and resistance genes, although few field-scale studies have examined the relative efficiency of each method. This study examined the ability of both methods in both winter-spring and summer-fall cycles, while also accounting for heterogeneity within field-scale manure piles. This study determined that bulking agents used in composting could contribute antibiotic-resistant bacteria and resistance genes. Additionally, seasonal variation could hinder the efficacy of composting in colder months due to heterogeneity in temperature within the pile; however, in warmer months, either method of manure storage could be effective in reducing the spread of antibiotic resistance.

**KEYWORDS** antibiotic resistance gene, antibiotic-resistant bacteria, beef cattle manure, composting, stockpiling



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The spread of antibiotic resistance is of growing public health concern, as many bacterial pathogens have developed resistance to routine antibiotics (1, 2) and drugs of last resort, such as carbapenems (3, 4). The spread of antibiotic resistance is partially

attributed to the use of antibiotics in livestock operations, which accounts for up to 80% by mass of total antibiotic usage in the United States (5, 6). Administration of antibiotics to livestock can increase antibiotic-resistant bacteria (ARB) as well as antibiotic resistance genes (ARGs) in livestock wastes (7, 8). Additionally, livestock wastes can be a reservoir for zoonotic pathogens such as *Escherichia coli* O157:H7 (9, 10), *Salmonella enterica* serovar Typhimurium (11), and *Listeria* and *Campylobacter* spp. (12). Consequently, the use of untreated livestock manure for land application may facilitate the spread of ARB, ARGs, and pathogens to soils and water (13–15). Soil and water receiving these microbial agents could facilitate their transmissions to human through contamination of food crops and direct exposure to the contaminated environment (16).

Hence, it is important that proper manure management strategies are used prior to land application. One common practice for manure storage is stockpiling (17, 18), through which pen surface materials are piled and the piles are allowed to sit and age until land application. Manure stockpiles can sometimes heat up initially; however, they may not reach a sufficiently high temperature or maintain the elevated temperature for a sufficient length of time to reduce ARG/ARGs. Hence, stockpiling may not be sufficient treatment to kill off zoonotic pathogens and reduce ARB/ARGs (10).

Composting is an alternative to stockpiling. According to the Food and Drug Administration Produce Safety Rule for application of soil amendments of animal origin, static composting must maintain aerobic conditions and temperatures of 55°C for 3 consecutive days, while turned composting must maintain 55°C for 15 cumulative days, with a minimum of five turning events (19). Similarly, Environmental Protection Agency guidelines require biosolids from municipal wastewater treatment facilities to be composted at 55°C and above for 3 days (20, 21). The heat generated during composting is derived from aerobic microbial activities. A bulking agent (e.g., corn stalk residues, wood chips, or sawdust) is commonly added to manure to increase oxygen penetration and meet microbial nutrient needs (22).

While composting is often considered more reliable than stockpiling to reduce pathogens in manure, it is also considerably more time-consuming, costly, and labor-intensive to establish and operate (23–25). The efficacy of composting for reducing ARB and ARGs has been investigated, mostly with lab-scale setups and some with field-scale piles (26). Although lab-scale reactors are simple to establish, they cannot adequately simulate full-scale composting piles. Hence, conclusions from lab-scale composting studies may not be directly applicable to field-scale composting piles. Field-scale studies have reported inconsistent results on the efficacy of composting on ARB/ARG reduction (26). Some field-scale studies have reported consistent decreases in ARB/ARG abundance during composting (27–31), while others have noted inconsistent effects (i.e., some ARGs decreased while others persisted or even increased) (32–35). This is not surprising because of the variability in manure characteristics, choice of bulking agents, ambient temperature, and pile operation, which could all affect the inactivation of ARB and reduction of ARGs. In addition, owing to the heterogeneity in full-scale manure piles, the sampling strategy employed to collect samples from manure piles can also affect the conclusions. To comprehensively assess the efficacy of composting in reducing ARB and ARGs in livestock manure, more full-scale studies with systematic sample strategies, to account for manure pile heterogeneity, are needed.

The objective of this study was to investigate the effectiveness of composting in reducing ARB and ARGs in beef cattle manure using field-scale experiments. This required an experimental design to account for the suspected heterogeneity inside the stockpiles and composting piles. Both the stockpile and composting piles were operated for the length of time required for two complete composting cycles. One cycle was operated during the winter-spring and one cycle was operated during the summer-fall, allowing for assessment of seasonal differences in full-scale systems. To better account for the piles, samples were collected at two or three profiles along each pile and at three depths per profile. Additionally, detailed temperature profile data were also documented from each pile. These measures were used to facilitate a more

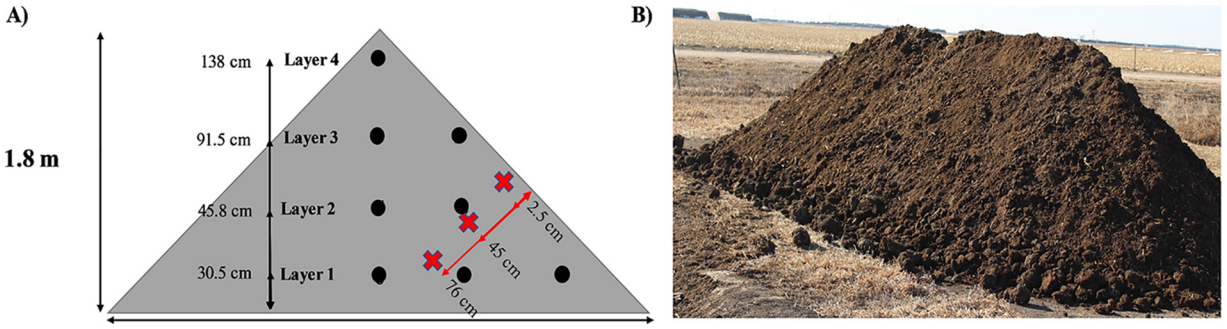


**FIG 1** (A) Placement of thermocouples and sampling locations within the composting piles and stockpiles. Black dots represent thermocouple placement, and red crosses represent sampling locations. (B) Photograph of one of the composting piles from the winter-spring cycle.

comprehensive and objective comparison between the stockpiling and composting methods.

## RESULTS

**The winter-spring cycle. (i) Temperature.** After pile establishment (16 December 2017), the interior temperatures of all four piles, two composting piles and two stockpiles, quickly exceeded 55°C (see Table S2 in the supplemental material). The two stockpiles exhibited temperatures above 55°C for about 15 days, mostly at layer 4 (138 cm from the base of the pile) of the piles (Fig. 1). In contrast, the two composting piles exhibited temperatures above 55°C in more layers and over a longer period (Fig. S2). For example, composting pile 2 retained temperatures exceeding 55°C in layers 2 to 4 for about 20 days. After the first turning (29 January 2018), both composting piles achieved temperatures exceeding 55°C in layers 2 to 4 for at least 5 more days. After the second turning (2 April 2018), both composting piles exhibited temperatures above 55°C in at least two layers for 9 days. The topmost layer in both composting piles stayed at temperatures above 55°C for 15 days (Table S2). The base of the piles never reached the desired temperature of 55°C, as the ground was frozen during the winter months.

**(ii) Bacterial concentrations and prevalence.** Initial concentrations of total *E. coli* and enterococci were higher in the pen scrapings than in the corn stalk residues prior to incorporation (Table 1). However, the corn stalk residues had the only quantifiable concentration of azithromycin-resistant (azithromycin$^R$) *E. coli*, which was not detected in the initial pen scrapings (Table 1). After the initial establishment of manure piles, the concentrations of total *E. coli* (Fig. S2) and total enterococci (Fig. S3) declined rapidly in the stockpiles. In all three depths of the stockpiles, total *E. coli* concentrations were undetectable after day 22, and total enterococcus concentrations were undetectable after day 43. In contrast, both bacteria continued to appear sporadically in the composting

**TABLE 1** Initial concentrations of total and antibiotic-resistant *E. coli* and enterococci in open feedlot pen scrapings and in the bulking agent for both cycles of the study

| Cycle and sample type | Initial concn (log CFU g$^{-1}$ [wet wt]) | | | | | |
|---|---|---|---|---|---|---|
| | Total *E. coli* | Azithromycin$^R$ *E. coli* | Tetracycline$^R$ *E. coli* | Total enterococci | Tylosin$^R$ enterococci | Tetracycline$^R$ enterococci |
| Winter-spring | | | | | | |
| Pen scrapings | 7.63 | BDL[a] | BDL | 11.63 | 1.38 | 2.63 |
| Corn stalk residues | 4.15 | 4.30 | BDL | 4.34 | BDL | 3.30 |
| Summer-fall | | | | | | |
| Pen scrapings | BDL | BDL | BDL | 3.78 | BDL | BDL |
| Rye silage | BDL | BDL | BDL | 4.93 | BDL | BDL |

[a]BDL, below detection limit.

**TABLE 2** Means and *P* values for storage method and depth for bacterial concentrations and bacterial prevalence in the winter-spring cycle[a]

| | Concn ($log_{10}$ CFU $g^{-1}$)[b] | | Prevalence (%) | | | | | |
|---|---|---|---|---|---|---|---|---|
| Parameter | Total *E. coli* | Total enterococci | Total *E. coli* | Azithromycin$^R$ *E. coli* | Tetracycline$^R$ *E. coli* | Total enterococci | Tylosin$^R$ enterococci | Tetracycline$^R$ enterococci |
| Storage method[c,d] | | | | | | | | |
| Composting | 1.45 A | 1.81 A | 58 A | 5 A | 10 A | 96 A | 82 A | 85 A |
| Stockpiling | 0.22 B | 0.32 B | 18 B | 1 B | 5 B | 62 B | 31 B | 27 B |
| Depth from surface (cm)[c,d] | | | | | | | | |
| 2.5 | 1.17 A | 1.54 A | 44 | 2 A | 13 A | 92 A | 69 A | 69 A |
| 45.7 | 0.76 B | 0.98 B | 38 | 6 B | 6 B | 86 A | 59 A | 58 A |
| 76.2 | 0.58 B | 0.68 B | 32 | 2 A | 5 B | 60 B | 42 B | 42 B |
| *P* value | | | | | | | | |
| Method | <0.001 | <0.001 | 0.002 | <0.001 | 0.031 | <0.001 | <0.001 | <0.001 |
| Depth | 0.008 | 0.001 | 0.113 | 0.016 | <0.001 | <0.001 | 0.003 | <0.001 |

[a]Statistics for interaction terms can be found in Table S3.
[b]Antibiotic-resistant *E. coli* and enterococci were not quantifiable.
[c]Values followed by a letter combination sharing one or more letters are not statistically different at the *P* value of <0.05 based on Tukey's *post hoc* test.
[d]Values are treatment averages which were calculated based on all data for one particular treatment level.

piles, particularly after each turning event (Fig. S2 and S3). Antibiotic-resistant *E. coli* and enterococci fell below the limit of quantification after the day 0 sampling event for all piles.

Analysis of variance (ANOVA) revealed that there was a significant main effect of manure storage method on total *E. coli* ($P < 0.001$) and total enterococcus ($P < 0.001$) (Table 2) concentrations. Contrary to expectation, higher concentrations of both bacteria were observed in the composting piles than in the stockpiles (Table 2). ANOVA also revealed a significant effect of depth for total *E. coli* and total enterococci ($P = 0.008$ and $P = 0.001$, respectively), with the highest concentrations in the 2.5-cm-depth samples. *Post hoc* analyses revealed that this was true only for composting piles.

Similarly, ANOVA results showed a significant effect of manure storage method on the prevalence of all bacterial populations tested. The frequency of detection was significantly greater for both *E. coli* and enterococci, total or resistant, in composting piles than in stockpiles ($P \leq 0.031$ for all cases [Table 2]; see Table S3 for more detailed statistics). The ANOVA also revealed a significant effect for depth on all but one bacterial population ($P \leq 0.016$ for all analyses except for total *E. coli* [Table 2 and Table S3]). In general, bacterial prevalence was significantly greater in 2.5-cm samples than in 76.2-cm samples.

The prevalence data for the two manure storage methods are illustrated in Fig. 2 and 3. The prevalence of both *E. coli* and enterococci, total and resistant, was consistently greater in composting piles than in stockpiles. Further, the detection frequency within the composting piles tended to increase followed turning events on days 49 and 112 (Fig. 2 and 3). Noticeably, at the end of the 140-day experiment, the total and resistant *E. coli* organisms were no longer detectable in the stockpiles but were still detected frequently in the composting piles.

**(iii) ARG concentrations.** Based on the ANOVA, there was no significant effect for manure storage methods for most of the genes tested, except for *intI1* ($P = 0.003$ [Table 3]). The *intI1* concentration was greater in the composting piles than in the stockpiles (Table 3 and Table S4). A significant effect of depth was also observed only for the 16S rRNA gene ($P = 0.045$) and *intI1* ($P = 0.005$). *Post hoc* tests indicate that this effect was driven by significantly greater concentrations at the 2.5-cm depth than at the 76.2-cm depth in stockpiles for the 16S rRNA gene ($P = 0.035$) and in both stockpiling and composting piles for *intI1* ($P = 0.045$ and 0.049, respectively [Fig. 4]). ANOVA revealed a significant time effect on the concentrations of all ARGs examined following the first turning on day 49 (29 January 2018) (Table S4). There was an increase in concentration for most ARGs, followed by a decrease of approximately 1 order of magnitude for the subsequent sampling event (Fig. 5).

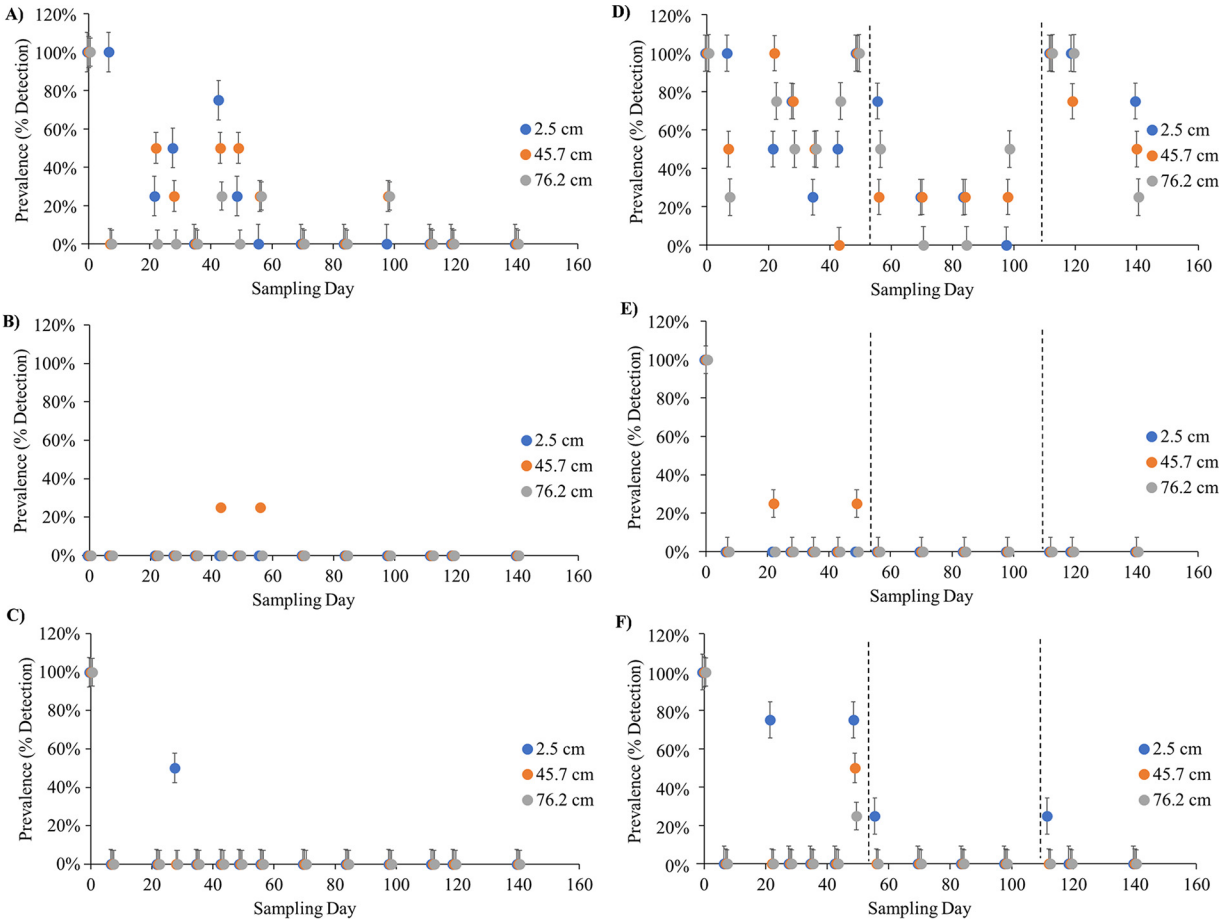


**FIG 2** Prevalence at three depths on each sampling event of total *E. coli* (A), azithromycin$^R$ *E. coli* (B), and tetracycline$^R$ *E. coli* (C) in stockpiles and total *E. coli* (D), azithromycin$^R$ *E. coli* (E), and tetracycline$^R$ *E. coli* (F) in the composting piles during the winter-spring cycle. Black lines (days 49 and 112) indicate dates on which composting piles were turned and samples were taken immediately after. Error bars represent standard errors ($n = 4$ profiles per sampling event).

**Summer-fall cycle. (i) Temperature.** Temperatures for nearly all measured locations within the composting pile were greater than 55°C for all 47 days of the summer-fall cycle (8 August 2018 to 24 September 2018 [Table S5]). Consequently, the composting pile was not turned during this cycle of the experiment. Layers 3 and 4 within the stockpile consistently exceeded 55°C for about 27 days (Table S5). However, temperatures measured near the bottom of the stockpile never exceeded 55°C (Table S5).

**(ii) Bacterial concentrations and prevalence.** Pen surface materials for the summer-fall cycle of the study had considerably lower bacterial concentrations than for the winter-spring cycle, with only total enterococci being detected above the limit of quantification (Table 1). The rye silage used as a bulking agent had no detectable ARB or total *E. coli* but had total enterococcus concentrations greater than those of the pen surface materials (Table 1). Similar to the case with the winter-spring cycle, resistant *E. coli* and enterococci were consistently below the limit of quantification throughout the study, and quantifiable total *E. coli* and enterococcus concentrations occurred sporadically in the stockpile and composting pile.

ANOVA results showed that the manure storage method had a significant impact on the concentration of total *E. coli* ($P = 0.019$ [Table 4]). Similar to what was found in the winter-spring cycle, total *E. coli* concentrations were higher in the composting pile than in the stockpile. Also, the manure storage method had a significant impact on the prevalence of resistant *E. coli*, total enterococci, and tetracycline$^R$ enterococci. For these four populations, there were significantly greater prevalences in the composting pile than in

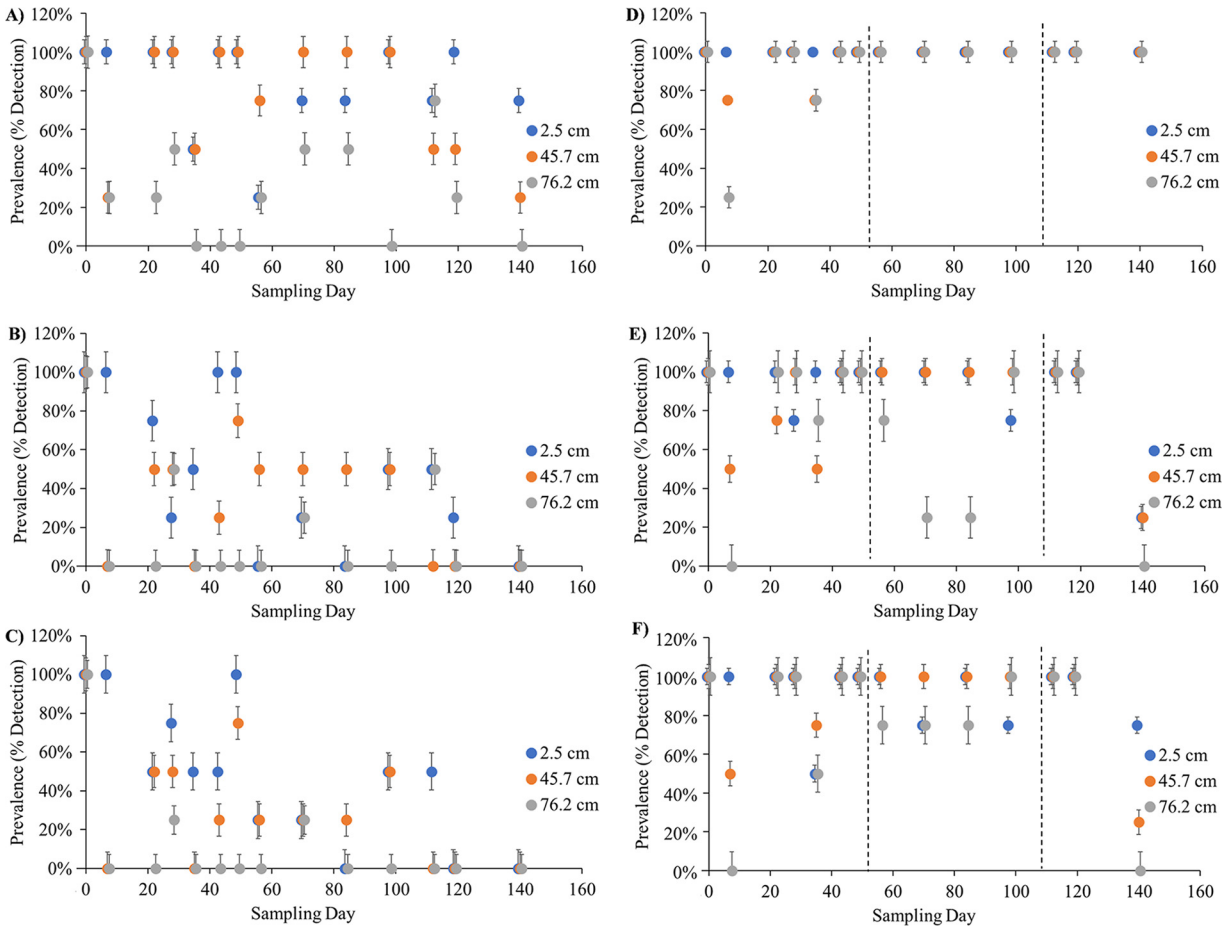


**FIG 3** Prevalence at three depths on each sampling event of total enterococci (A), tylosin$^R$ enterococci (B), and tetracycline$^R$ enterococci (C) in stockpiles and total enterococci (D), tylosin$^R$ enterococci (E), and tetracycline$^R$ enterococci (F) in the composting piles during the winter-spring cycle. Black lines (days 49 and 112) indicate dates on which composting piles were turned and samples were taken immediately after. Error bars represent standard errors ($n = 4$ profiles per sampling event).

the stockpile (Fig. 6 and 7 and Table 4; see Table S6 for more detailed statistics). Depth did not appear to significantly impact the concentrations or the prevalence of the tested bacterial populations.

**(iii) ARG concentrations.** ANOVA results indicate that the manure storage method had no significant effect on the genes tested, except *erm*(F). The *erm*(F) gene was significantly lower in the composting pile than in the stockpile (Table 5 and Table S7). A significant main effect for depth was detected for *tet*(Q) ($P = 0.005$). The *tet*(Q) gene concentration was significantly greater at the 76.2-cm depth than at the 45.7-cm and 2.5-cm depths ($P = 0.009$ and 0.007, respectively [Fig. 8]). Also, ANOVA results indicated a significant time effect for the four ARGs (Table S7). For *erm*(B), *tet*(O), and *tet*(Q), this effect was driven by an overall decline in concentrations in both the stockpile and composting pile (Fig. 9), while for *erm*(F), no overall decline was noted and this effect was driven by an increase in concentrations on 27 August 2018 (day 19).

## DISCUSSION

A review of the available literature showed a limited number of full-scale studies evaluating the effect of composting of material removed from open-lot beef feedlots on ARB and ARGs. Some studies reported effective reduction of ARB and ARGs during composting (28, 36, 37), while others reported inconsistent or contrary results (32–35). Among previous field-scale studies, differences in manure composition (e.g., caused by species, feed, etc.) and composting process (e.g., bulking agents) are likely responsible for the

**TABLE 3** Means and *P* values for storage method and depth for ARG concentrations in the winter-spring cycle[a]

| | Concn ($log_{10}$ CN $g^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| **Parameter** | **16S rRNA gene** | ***intI1*** | ***erm*(B)** | ***erm*(F)** | ***tet*(O)** | ***tet*(Q)** |
| Storage method[b,c] | | | | | | |
| Composting | 9.62 | 7.77 A | 4.93 | 6.88 | 4.33 | 6.01 |
| Stockpiling | 9.31 | 7.37 B | 4.53 | 6.13 | 3.95 | 5.55 |
| Depth from surface (cm)[b,c] | | | | | | |
| 2.5 | 9.60 A | 7.74 A | 4.72 | 6.85 | 4.19 | 5.94 |
| 45.7 | 9.41 B | 7.60 B | 4.78 | 6.42 | 4.17 | 5.90 |
| 76.2 | 9.38 AB | 7.36 B | 4.70 | 6.24 | 4.06 | 5.49 |
| *P* value | | | | | | |
| Method | 0.262 | 0.003 | 0.333 | 0.245 | 0.296 | 0.326 |
| Depth | 0.045 | 0.005 | 0.721 | 0.225 | 0.909 | 0.117 |
| Time | 0.023 | 0.007 | 0.027 | 0.022 | 0.004 | 0.009 |

[a]Statistics for interaction terms can be found in Table S4.
[b]Values followed by a letter combination sharing one or more letters are not statistically different at the *P* value of $<0.05$ based on Tukey's *post hoc* test.
[c]Values are treatment averages which were calculated based on all data for one particular treatment level.

seemingly contradictory findings. More importantly, the heterogeneity of field-scale manure piles may or may not be sufficiently accounted for by sampling strategies. Therefore, this study was designed to account for the heterogeneities by including two complete composting cycles using different manure, two distinct seasons, and monitoring/sampling multiple locations for temperature and ARB/ARGs throughout the manure piles.

**Effects on temperature.** As expected, the internal temperatures of composting piles were higher than those of stockpiles (Tables S2 and S5). Also, there was less heterogeneity

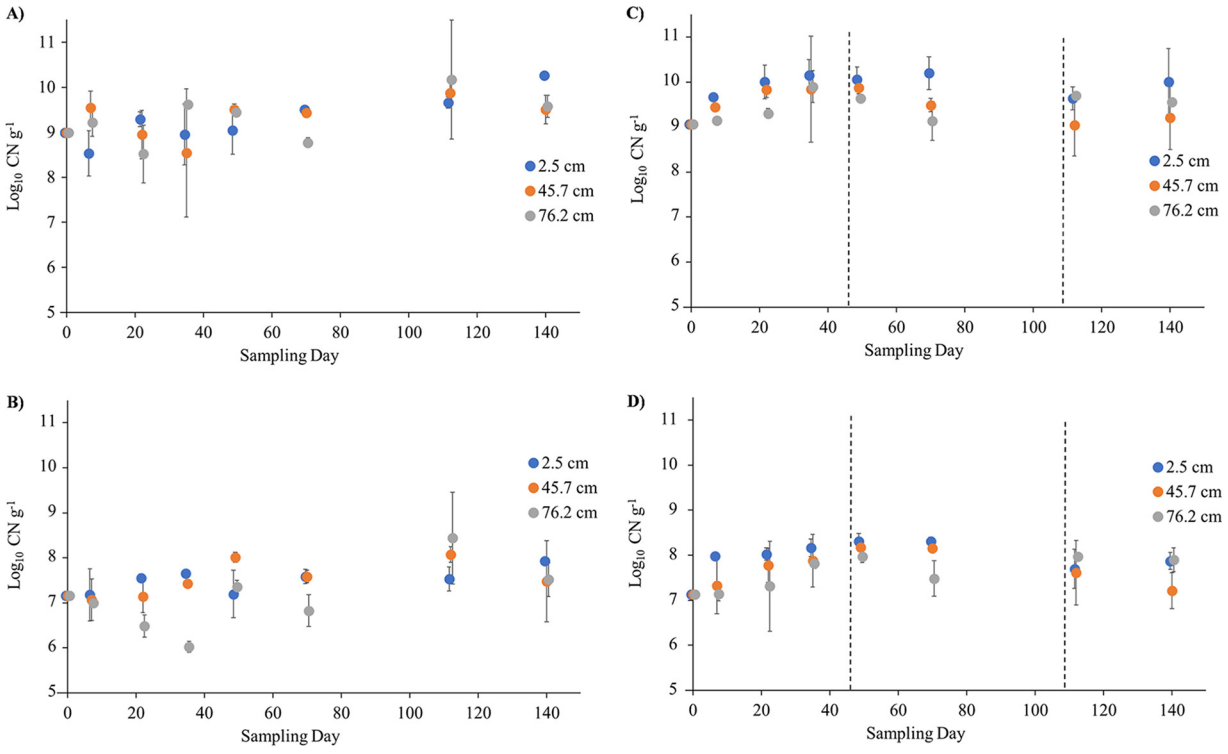


**FIG 4** Concentrations at three depths on each sampling event of the 16S rRNA gene (A) and *intI1* (B) in a stockpile and the 16S rRNA gene (C) and *intI1* (D) in a composting pile during the winter-spring cycle. Black lines (days 49 and 112) indicate dates on which composting piles were turned and samples were taken immediately after. Error bars represent standard deviations ($n = 2$ profiles per sampling event).

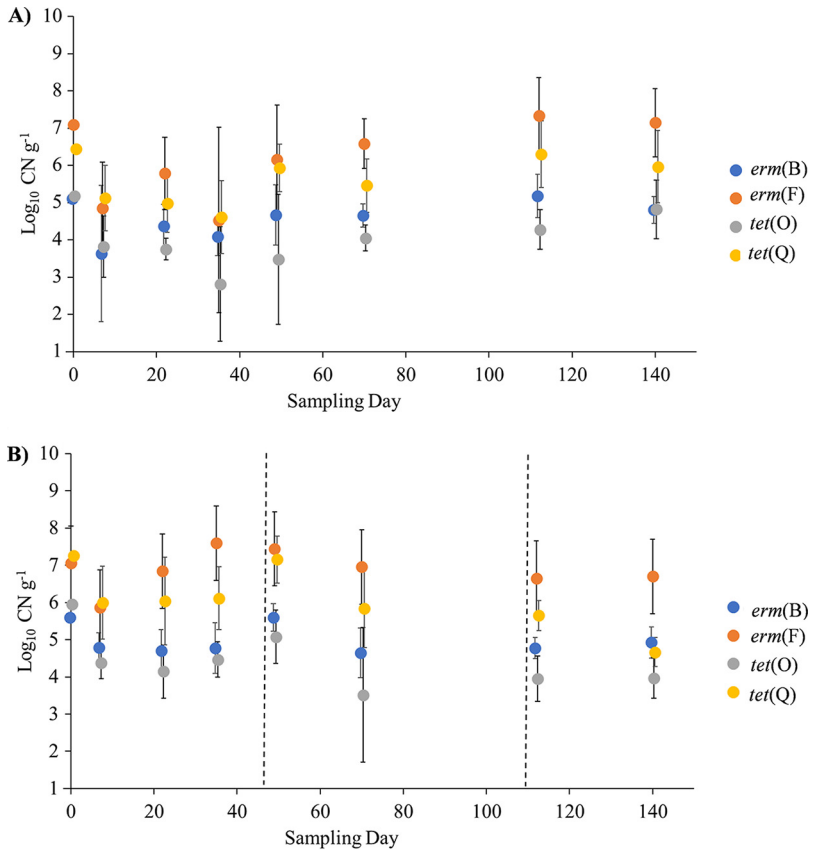


**FIG 5** Concentrations of ARGs for a stockpile (A) and a composting pile (B) over time during the winter-spring cycle. Black lines (days 49 and 112) indicate dates on which compost piles were turned and samples were taken immediately after. Error bars represent standard errors ($n = 6$, 2 profiles × 3 depths per sampling event).

in the temperature profiles of both composting and stockpiles in the summer-fall cycle than in the winter-spring cycle. This was due to elevated atmospheric and ground temperatures. The mean monthly ambient temperatures were −2.8°C, −4.7°C, −5.3°C, 3.9°C, and 5.8°C from December to April during the winter-spring cycle and 22.5°C and 19.2°C in

**TABLE 4** Means and *P* values for storage method and depth for bacterial concentrations and bacterial prevalence in the summer-fall cycle[a]

| | Concn ($log_{10}$ CFU $g^{-1}$)[b] | | Prevalence (%) | | | | | |
|---|---|---|---|---|---|---|---|---|
| Parameter | Total *E. coli* | Total enterococci | Total *E. coli* | Azithromycin$^R$ *E. coli* | Tetracycline$^R$ *E. coli* | Total enterococci | Tylosin$^R$ enterococci | Tetracycline$^R$ enterococci |
| Storage method[c,d] | | | | | | | | |
| Composting | 0.66 A | 0.85 | 54 | 18 A | 13 A | 87 A | 23 | 36 A |
| Stockpiling | 0.52 B | 0.82 | 49 | 3 B | 0 B | 67 B | 19 | 17 B |
| Depth from surface (cm)[c,d] | | | | | | | | |
| 2.5 | 0.80 | 0.98 | 61 | 15 | 8 | 88 | 29 | 33 |
| 45.7 | 0.61 | 0.74 | 49 | 8 | 4 | 80 | 21 | 29 |
| 76.2 | 0.33 | 0.78 | 45 | 8 | 8 | 64 | 13 | 17 |
| *P* value | | | | | | | | |
| Method | 0.019 | 0.518 | 0.462 | 0.001 | <0.001 | <0.001 | 0.060 | 0.002 |
| Depth | 0.265 | 0.594 | 0.477 | 0.198 | 0.683 | 0.120 | 0.198 | 0.256 |

[a]Statistics for interaction terms can be found in Table S6.
[b]Antibiotic-resistant forms of *E. coli* and enterococci were not quantifiable.
[c]Values followed by a letter combination sharing one or more letters are not statistically different at the *P* value of <0.05 based on Tukey's *post hoc* test.
[d]Values are treatment averages which were calculated based on all data for one particular treatment level.

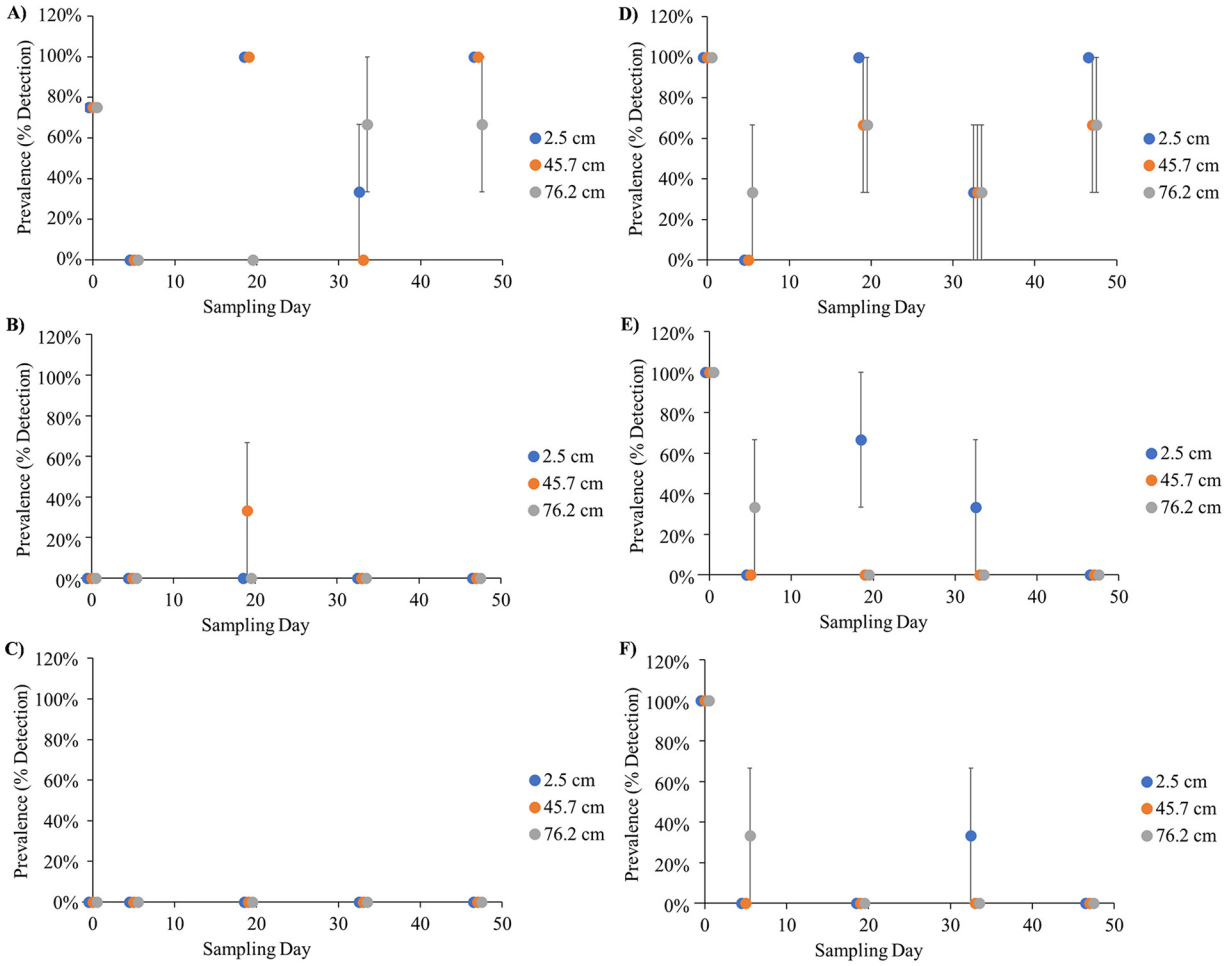


**FIG 6** Prevalence at three depths on each sampling event of total *E. coli* (A), azithromycin$^R$ *E. coli* (B), and tetracycline$^R$ *E. coli* (C) in the stockpile and total *E. coli* (D), azithromycin$^R$ *E. coli* (E), and tetracycline$^R$ *E. coli* (F) in the composting pile during the summer-fall cycle. Error bars represent standard errors ($n = 3$ profiles per sampling event).

August and September during the summer-fall cycle. In the summer-fall cycle, temperatures exceeding 55°C were achieved nearly everywhere in the composting pile. In contrast, in the winter-spring cycle temperatures exceeding 55°C were reached only in upper portion of the composting piles due to frozen ground.

**Effects on bacteria.** Our data suggest that certain bulking agents could introduce additional ARB to composting piles and that these elevated ARB concentrations could persist. Compared to their stockpiling counterparts, the composting piles in both cycles displayed greater prevalence of ARB (Tables 2 and 4). During the winter-spring cycle, the corn stalk residues used as the bulking agent contained high concentrations of ARB, including azithromycin$^R$ *E. coli*, which was absent in pen scrapings (Table 1). A previous survey showed ARB are commonly found on corn stalk residues, with ARB detected in 54% of corn stalk residues surveyed in Nebraska (38). Unlike the corn stalk residues used in the winter-spring round, the bulking agent rye silage used in the summer-fall cycle contained no quantifiable ARB (Table 1). However, *post hoc* analyses found significantly greater prevalence of azithromycin$^R$ *E. coli*, tetracycline$^R$ *E. coli*, total enterococci, and tetracycline$^R$ enterococci in the composting pile than in the stockpile (Table 4). This is likely attributable to rye silage. As the bulking agents were mixed with pen scrapings at a 40:60 ratio, they could represent a considerable source of ARB to the composting piles. Hence, choosing a bulking agent free of ARB could be important to achieving ARB control in composting piles. It is noted that in the summer-fall round,

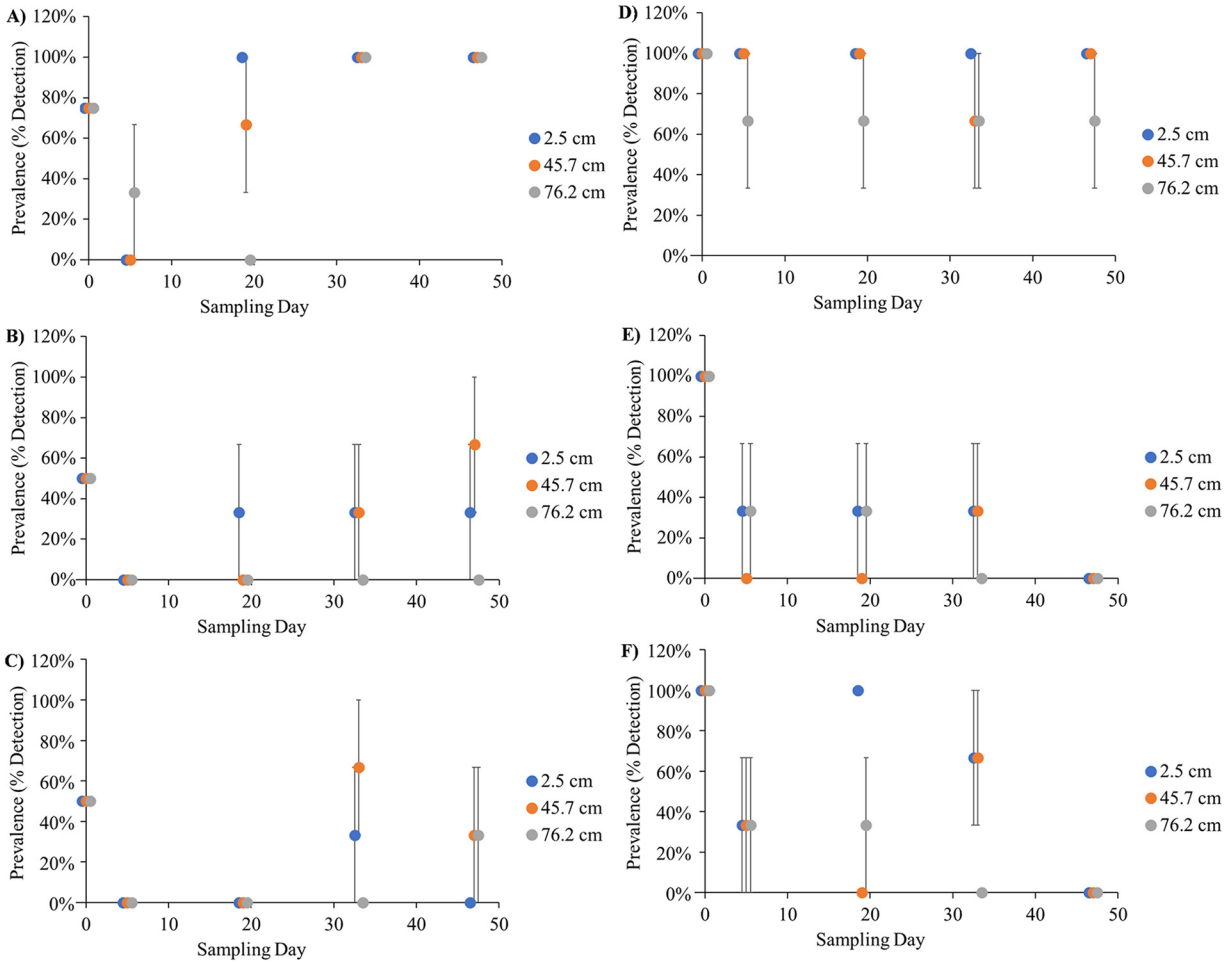


**FIG 7** Prevalence at three depths on each sampling event of total enterococci (A), tylosin$^R$ enterococci (B), and tetracycline$^R$ enterococci (C) in the stockpile and total enterococci (D), tylosin$^R$ enterococci (E), and tetracycline$^R$ enterococci (F) in the composting pile during the summer-fall cycle. Error bars represent standard errors ($n = 3$ profiles per sampling event).

both pen scrapings and rye silage initially had *E. coli* concentrations below the limit of detection. However, quantifiable *E. coli* concentrations were later found in both piles, suggesting the possibility of growth of *E. coli* in portions of the manure piles during both stockpiling and composting.

Turning events appeared to cause transient increases in ARB concentration in composting piles. Turning could stimulate microbial activities by redistributing nutrients and replenishing oxygen inside manure piles, sustaining the heating of composting piles (39). During the winter-spring cycle with the low atmospheric temperature and frozen ground, portions of the composting pile never exceeded 55°C. Instead, temperatures favorable to bacterial growth (~35 to 45°C) (40, 41) occurred in the lower section of the piles sitting on frozen ground. In contrast, the base of the stockpiles remained at very low temperatures throughout the experiment. During turning events, materials that previously resided in portions where temperatures favorable to bacterial growth occurred were mixed with materials from other portions of the manure piles and served as bacterial seed. This may explain why there were transient increases in bacterial concentration (Fig. S2 and S3) and prevalence (Fig. 2 and 3) after turning.

In order to identify common trends between the relationship of temperature and prevalence of indicator bacteria under the two treatment regimes, we combined the data from the winter-spring round and the summer-fall round of experiments. When all data points were pooled, it was found that there were consistently higher

**TABLE 5** Means and *P* values for storage method and depth for ARG concentrations in the winter-spring cycle[a]

| | Concn ($log_{10}$ CN $g^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| **Parameter** | **16S rRNA** | ***intI1*** | ***erm*(B)** | ***erm*(F)** | ***tet*(O)** | ***tet*(Q)** |
| Storage method[b,c] | | | | | | |
| Composting | 9.35 | 7.15 | 4.31 | 6.25 A | 4.66 | 5.33 |
| Stockpiling | 9.52 | 7.39 | 4.78 | 6.98 B | 4.91 | 5.70 |
| Depth from surface (cm)[b,c] | | | | | | |
| 2.5 | 9.46 | 7.38 | 4.54 | 6.87 | 4.55 | 5.25 A |
| 45.7 | 9.52 | 7.40 | 4.45 | 6.39 | 4.69 | 5.31 A |
| 76.2 | 9.31 | 7.04 | 4.65 | 6.58 | 5.11 | 5.97 C |
| *P* value | | | | | | |
| Method | 0.367 | 0.508 | 0.162 | 0.013 | 0.447 | 0.472 |
| Depth | 0.106 | 0.083 | 0.377 | 0.053 | 0.106 | 0.005 |
| Time | 0.159 | 0.393 | <0.001 | 0.002 | <0.001 | <0.001 |

[a]Statistics for interaction terms can be found in Table S7.
[b]Values followed by a letter combination sharing one or more letters are not statistically different at the *P* value of <0.05 based on Tukey's *post hoc* test.
[c]Values are treatment averages which were calculated based on all data for one particular treatment level.

prevalences of total and resistant *E. coli* and enterococci under composting treatment than under stockpiling treatment (Fig. S4A and C). When only data points with measured temperatures at or above 55°C were considered, the distinctions in prevalence between composting and stockpiling become much less pronounced (Fig. S4B and D). These findings suggest that temperature above 55°C is a reliable predictor for the inactivation of total and resistant indicator bacteria regardless of treatment regime. Furthermore, when bulking agents potentially contribute to the total and resistant indicator bacteria, the portions of composting piles with temperatures below 55°C become the "hot spots" of these microbes.

**Effects on ARGs.** No obvious advantage of composting was observed with respect to ARG reduction in either cycle of this study. Neither stockpiling nor composting

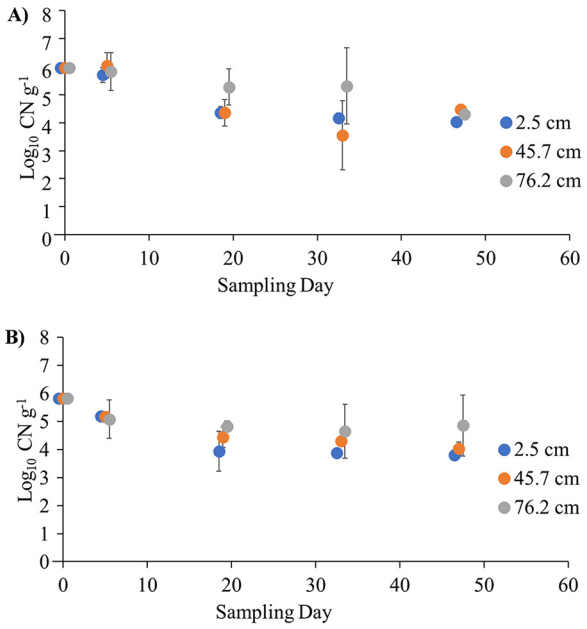


**FIG 8** Concentrations of *tet*(Q) at each sampling depth from two profiles on each sampling day of the summer-fall cycle in the stockpile (A) and the composting pile (B). Error bars represent standard deviations ($n = 2$ profiles per sampling event).

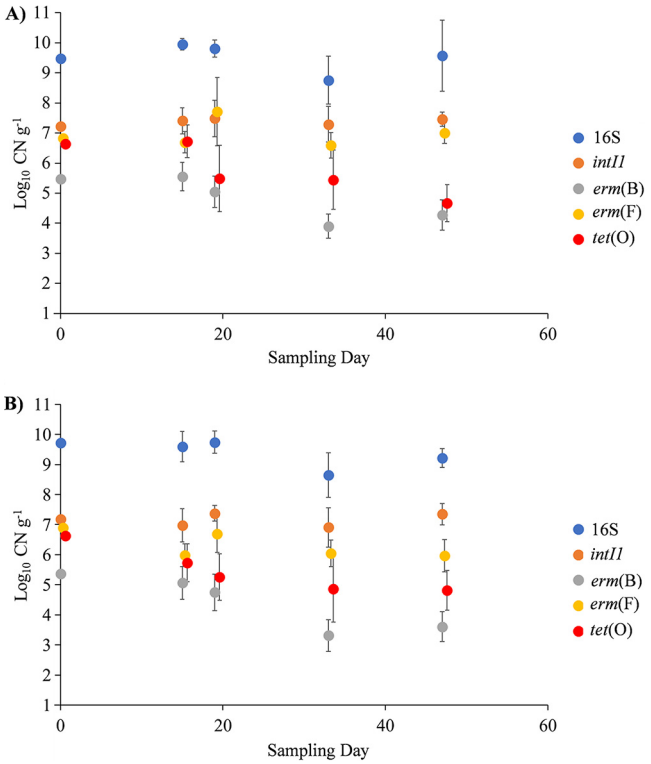


**FIG 9** Concentrations of the 16S rRNA gene, *intI1*, and three ARGs from two profiles for each manure storage method over time during the summer-fall cycle in the stockpile (A) and the composting pile (B). Error bars represent standard errors ($n = 6$; 2 profiles × 3 depths per sampling event).

showed a significant reduction in ARGs when their final concentrations were compared with the initial concentrations during the winter-spring cycle according to *post hoc* analyses, despite fluctuating ARG concentrations over time (Table 3). Because the ARG reduction performance was poor for both stockpiling and composting, there was no significant difference between the two methods (Table 3 and Table S4). In contrast, during the summer-fall cycle, *post hoc* analyses showed that the final concentrations of *erm*(B) and *tet*(O) were significantly lower than the initial concentrations in both stockpiles ($P = 0.020$ and 0.007, respectively) and composting piles ($P = 0.007$ and 0.038, respectively). Because a significant reduction occurred in both stockpiles and composting piles, the difference between the two storage methods was not significant (Table 5).

The fate of ARGs in manure composting piles is likely linked to the fate of their bacterial hosts, based on their thermotolerance (28, 36, 42). Composting with sawdust (30) and red mud (29) as bulking agents could result in reduction of ARGs by stimulating the growth and activity of certain microbial populations in composting piles (43, 44). Inconsistent reduction in ARGs and alterations in the microbial community were noted in a field-scale study composting cattle manure with straw as a bulking agent (32). In that 2016 study by Qian et al., the ARGs *tet*(C) and *tet*(X) increased, while *tet*(Q), *tet*(M), and *tet*(W) decreased, as different bacterial community profiles were found to be associated with changes in the ARG profile as a factor of composting time. Another field-scale composting study in which hyperthermophilic temperatures (>90°C) were reached similarly found significant reductions in *tet* genes, associating the reduction with the removal of thermotolerant bacterial species and associated mobile genetic elements (27). Several studies have also linked ARG expression with changes in microbial community composition during the composting process. For example, *Firmicutes* were the most transcriptionally active ARG carriers during the mesophilic phase (~40°C) of composting,

while *Firmicutes*, *Actinobacteria*, *Bacteroides*, and *Proteobacteria* were the most transcriptionally active ARG carriers during the thermophilic phase (>60°C) of the composting, in one study (45). Consequently, despite notable declines in less thermotolerant bacterial taxa, ARG abundance can remain the same as antibiotic-resistant thermotolerant taxa thrive or obtain ARGs via horizontal gene transfer under thermophilic conditions (46).

Compared to stockpiling, composting did not consistently reduce ARB or ARGs in the open feedlot pen scrapings in this study. The use of a bulking agent in composting piles appeared to increase ARB concentrations and prevalence due to the introduction of additional ARB from bulking agents. The heterogeneity in temperature profiles, caused by low ambient air temperature and frozen ground in winter and spring months, also contributed to increased ARB prevalence in composting piles by creating temperature gradients that were favorable to bacterial growth. Composting did not exhibit superior performance on ARG reduction over stockpiling, as no ARG was significantly reduced under either method in the winter-spring cycle and was significantly reduced under both methods in the summer-fall cycle. Results from this study suggest that (i) bulking agent can be an important source of ARGs for composting; (ii) during cold months, the heterogeneity of the temperature profile in composting piles could result in poor ARG reduction; and (iii) during warm months, both stockpiling and composting can be effective in reducing ARG abundance.

## MATERIALS AND METHODS

**Setup and sampling of composting and stockpiles. (i) Winter-spring.** Two composting piles and two stockpiles were established at the USDA U.S. Meat Animal Research Center (MARC) near Clay Center, NE. The stockpiles consisted of pen scrapings from beef cattle feedlot pens, while the composting piles consisted of a 60:40 ratio of pen scrapings to ground corn stalks, which were used as the bulking agent. This ratio was determined based on literature values for the carbon and nitrogen levels in cattle manure and corn stalk residues to achieve a final C/N ratio of 30:1 in compost piles (47). All four piles were approximately 6.1 m long, 3.0 m wide, and 1.8 m high. All four piles were set up on 11 December 2017 and operated until 30 April 2018. Composting piles were turned by physically moving the piles to an adjacent site 3 m to the side using a Bobcat front loader after day 49 (29 January 2018) and day 112 (2 April 2018), when the interior temperature dropped and remained below 50°C. Within each composting and stockpile, eight type-T thermocouples (Omega Engineering, Inc., Norwalk, CT) were placed along four horizontal layers within a profile (Fig. 1), with vertical distances 30.5, 45.8, 91.5, and 138 cm from the ground, to record temperature hourly using an OM-CP-OCTTemp A 8-channel multiplexer/data logger (Omega Engineering, Inc.).

Samples were collected from the four piles after the initial establishment and on days 7, 22, 28, 35, 43, 49, 56, 70, 84, 98, 112, 119, and 140. A grab sample of the corn stalk, prior to being incorporated into the composting piles, was collected for ARB analysis. To account for the potential heterogeneity along the manure piles, samples were collected from two profiles per pile at all sampling intervals. At each sampling interval, the sampling profiles were moved 0.15 m away from the immediate past sampling event. At each sampling profile, samples were collected from three depths from pile surface, 2.5 cm, 45.7 cm, and 76.2 cm, into the center of the pile (Fig. 1). Hence, six samples (2 profiles × 3 depths) were collected per pile per sampling event. Approximately 50 g of material was obtained at each depth. Samples at the 2.5-cm depth were collected via grab sampling, while those at the 45.7- and 76.2-cm depths were collected using an ethanol-sterilized push probe. Only the bottom section of manure in the push probe was recovered to avoid bacterial contamination from the superficial layer. All samples were placed in sterile 50-ml centrifuge tubes, stored on ice, and transported to the environmental engineering lab at the University of Nebraska—Lincoln (UNL) within 2 h of collection.

**(ii) Summer-fall.** During the second cycle of study, a composting pile and a stockpile were established and operated at MARC from 8 August 2018 through 24 September 2018. The stockpile similarly consisted of pen scrapings, while the composting pile consisted of a 60:40 ratio of pen scraping and rye silage, used as the bulking agent. Pile dimensions and thermocouple placement were the same as for the first cycle. The interior temperature of the composting pile never dropped below 50°C; hence, the composting pile was not turned during the study period.

The sampling procedure for the winter-spring cycle was largely adopted for the summer-fall cycle, with some modifications. Samples were collected after initial pile establishment and at days 5, 19, 33, and 47. Instead of two, three profiles were sampled per pile per sampling event. Within each sampling profile, samples from the same three depths as the first cycle were collected. A total of nine samples (3 profiles × 3 depths) were collected per pile per sampling event. A sample of rye silage was taken for ARB analysis prior to incorporation with pen scrapings. All samples were placed in polyethylene bags, stored on ice, and transported to the UNL environmental engineering lab within 2 h of collection.

**ARB enumeration and detection.** All samples were assessed for abundance and presence of total culturable *E. coli* and enterococci, as well as culturable forms of both bacteria with resistance to a

macrolide antibiotic (azithromycin and tylosin, respectively) and tetracycline. Tetracycline and tylosin are veterinary antibiotics commonly used on beef cattle and were used at the MARC facility (48–50). Tylosin and azithromycin both belong to the macrolide antibiotic family. Because *E. coli* is intrinsically resistant to tylosin, azithromycin was used to screen for macrolide-resistant *E. coli* (51). All solid samples were suspended 1:10 (wt/vol) in phosphate-buffered tryptic soy broth (Becton, Dickinson, Sparks, MD; TSB-$PO_4$), shaken vigorously for 2 min to dislocate bacteria associated with the samples, and plated for enumeration. *E. coli* organisms were enumerated on MacConkey agar (Becton, Dickinson) with no antibiotic, 20 mg liter$^{-1}$ of azithromycin, or 32 mg liter$^{-1}$ of tetracycline, while enterococci were quantified on Slanetz-Bartley agar (Oxoid, Hampshire, England) with no antibiotics, 32 mg liter$^{-1}$ of tylosin, or 32 mg liter$^{-1}$ of tetracycline (48–50). Concentrations of antibiotics were selected based on previously described clinical breakpoints and results with cattle manure (51, 52). An Eddy Jet 2 spiral plater (IUL, S.A., Barcelona, Spain) was used for plating. MacConkey plates were incubated at 37°C for 24 h, and Slanetz-Bartley plates were incubated at 44°C for 48 h. Quantifiable results are reported as CFU per gram of composted or stockpiled materials. Colony counting was performed using spiral counting grids provided by IUL, S.A., made specifically for the Eddy Jet 2 as per the instrument instruction manual, with a limit of quantification of 1 CFU 100 $\mu$l$^{-1}$ of suspension.

To assess the presence/absence of each bacterial target, all samples were subjected to a 24-h enrichment step for prevalence assessment. *E. coli* organisms were enriched in TSB-$PO_4$ and enterococci were enriched in Enterococcosel broth (Becton, Dickinson, Sparks, MD), both at 37°C for 24 h, prior to the same plating and incubation procedures as mentioned above.

To verify that colonies grown on MacConkey agar were *E. coli*, 20 isolates from one sampling event were selected and incubated in nutrient broth with 4-methylumbelliferyl-$\beta$-D-glucuronide (Difco, Sparks, MD). All were confirmed to be *E. coli* according to EPA method 9221F (53). An additional 426 colonies were also verified as *E. coli* via matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS; Shimadzu, Kyoto, Japan).

**Propidium monoazide DNA extraction.** A subset of samples was chosen for ARG analyses from one composting and one stockpile in the winter-spring cycle (i.e., days 0, 7, 22, 35, 49, 70, 112, and 140) and from one composting and one stockpile in the summer-fall cycle (i.e., days 0, 5, 19, 33, and 47). A modified DNA extraction protocol involving propidium monoazide (PMA) was optimized for composted and stockpiled materials (Fig. S1) and employed in this study.

The modified DNA extraction procedure involved weighing out 0.1 g of sample, suspending it in 0.9 ml of autoclaved ultrapure water, and vortexing the suspension for 30 s. Fifteen microliters of 20 mM PMA was then added to the suspension for a final PMA concentration of 300 $\mu$M. Samples were then incubated in the dark for 5 min with shaking prior to being transferred to sterile Pyrex petri dishes. Petri dishes were placed on ice and exposed to a 500-W halogen light 15 cm away for 30 min (54, 55). The ice was replaced every 10 min. Following light exposure, the suspension was transferred to a PowerBead tube and DNA extraction was performed using a DNeasy PowerLyzer PowerSoil kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions.

**ARG quantification.** Quantitative PCR (qPCR) was performed to quantify the 16S rRNA gene (56) and the *intI1* gene (57), as well as macrolide resistance genes *erm*(B) and *erm*(F) (58) and tetracycline resistance genes *tet*(O) and *tet*(Q) (59). These genes were selected because of their prevalence in agricultural environments and their previous detection in manure obtained from MARC (58, 60–62). Each reaction consisted of 2× KiCqStart universal SYBR green ReadyMix (Sigma-Aldrich, St. Louis, MO) and final primer concentrations of 0.3 $\mu$M for genes *intI1* and *tet*(Q), 0.375 $\mu$M for the 16S rRNA gene, 0.4 $\mu$M for *erm*(B), and 0.5 $\mu$M for genes *erm*(F) and *tet*(O). Reactions were carried out in duplicates, including no-template controls, on an Eppendorf Realplex$^2$ thermocycler (Eppendorf, Hamburg, Germany). Cycling conditions for each gene are presented in Table S1. Standard curves were run on all plates for all qPCR assays and were constructed using synthesized plasmid DNA (pIDTSMART with ampicillin resistance; Integrated DNA Technologies, Coralville, IA). DNA used for the standard curve was serially diluted in PCR-grade reagent water (Sigma-Aldrich). All qPCR runs had an efficiency between 90% and 105%, with an $R^2$ of $>$0.95. Results were reported as copy number (CN) per gram (wet weight) of manure or compost.

**Statistical analyses.** For results from culture-based analyses, ARB were detected at quantifiable concentrations only immediately following pile establishment in both cycles and were therefore not included in the statistical analyses. With regard to bacterial prevalence (presence/absence), a split-plot ANOVA was conducted with manure storage method (i.e., composting versus stockpiling) as the main treatment factor and depth as a repeated-measure factor. Time was not included as a repeated-measure factor due to sporadic detection, and consequently, each time point was considered a replicate for storage method and depth. The response variable was the total number of detections of total and resistant *E. coli* and enterococci over the course of the study. Tukey's *post hoc* test was performed if a significant effect was detected.

For results from qPCR analyses, split/split-plot ANOVA was conducted with manure storage method (i.e., composting versus stockpiling) as the main treatment factor and both depth and time as repeated measures providing a split-plot-in-time type of analysis. $Log_{10}$-transformed gene concentrations were response variables. For both cycles, gene analyses were conducted on two profiles from each manure pile and these profiles were treated as pseudoreplicates. All statistical analyses were performed in Statistica (Dell, Tulsa, OK) v.12, and results were considered significant at the level of 0.05.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.4 MB.

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## REFERENCES

1. Wright GD. 2007. The antibiotic resistome: the nexus of chemical and genetic diversity. Nat Rev Microbiol 5:175–186. https://doi.org/10.1038/nrmicro1614.
2. Marston HD, Dixon DM, Knisely JM, Palmore TN, Fauci AS. 2016. Antimicrobial resistance. JAMA 316:1193–1204. https://doi.org/10.1001/jama.2016.11764.
3. Pitout JDD, Laupland KB. 2008. Extended-spectrum $\beta$-lactamase-producing Enterobacteriaceae: an emerging public-health concern. Lancet Infect Dis 8:159–166. https://doi.org/10.1016/S1473-3099(08)70041-0.
4. Nordmann P, Dortet L, Poirel L. 2012. Carbapenem resistance in *Enterobacteriaceae:* here is the storm! Trends Mol Med 18:263–272. https://doi.org/10.1016/j.molmed.2012.03.003.
5. Boeckel TPV, Brower C, Gilbert M, Grenfell BT, Levin SA, Robinson TP, Teillant A, Laxminarayan R. 2015. Global trends in antimicrobial use in food animals. Proc Natl Acad Sci U S A 112:5649–5654. https://doi.org/10.1073/pnas.1503141112.
6. Mathew AG, Cissell R, Liamthong S. 2007. Antibiotic resistance in bacteria associated with food animals: a United States perspective of livestock production. Foodborne Pathog Dis 4:115–133. https://doi.org/10.1089/fpd.2006.0066.
7. Marshall BM, Levy SB. 2011. Food animals and antimicrobials: impacts on human health. Clin Microbiol Rev 24:718–733. https://doi.org/10.1128/CMR.00002-11.
8. Xie W-Y, Shen Q, Zhao FJ. 2018. Antibiotics and antibiotic resistance from animal manures to soil: a review. Eur J Soil Sci 69:181–195. https://doi.org/10.1111/ejss.12494.
9. Berry ED, Woodbury BL, Nienaber JA, Eigenberg RA, Thurston JA, Wells JE. 2007. Incidence and persistence of zoonotic bacterial and protozoan pathogens in a beef cattle feedlot runoff control vegetative treatment system. J Environ Qual 36:1873–1882. https://doi.org/10.2134/jeq2007.0100.
10. Berry ED, Millner PD, Wells JE, Kalchayanand N, Guerini MN. 2013. Fate of naturally occurring *Escherichia coli* O157:H7 and other zoonotic pathogens during minimally managed bovine feedlot manure composting processes. J Food Prot 76:1308–1321. https://doi.org/10.4315/0362-028X.JFP-12-364.
11. Semenov AV, van Overbeek L, van Bruggen AHC. 2009. Percolation and survival of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in soil amended with contaminated dairy manure or slurry. Appl Environ Microbiol 75:3206–3215. https://doi.org/10.1128/AEM.01791-08.
12. Nicholson FA, Groves SJ, Chambers BJ. 2005. Pathogen survival during livestock manure storage and following land application. Bioresour Technol 96:135–143. https://doi.org/10.1016/j.biortech.2004.02.030.
13. Joy SR, Bartelt-Hunt SL, Snow DD, Gilley JE, Woodbury BL, Parker DB, Marx DB, Li X. 2013. Fate and transport of antimicrobials and antimicrobial resistance genes in soil and runoff following land application of swine manure slurry. Environ Sci Technol 47:12081–12088. https://doi.org/10.1021/es4026358.
14. Chapman B, Pintar K, Smith BA. 2018. Multi-exposure pathway model to compare *Escherichia coli* O157 risks and interventions. Risk Anal 38:392–409. https://doi.org/10.1111/risa.12826.
15. Bicudo JR, Goyal SM. 2003. Pathogens and manure management systems: a review. Environ Technol 24:115–130. https://doi.org/10.1080/09593330309385542.
16. Marti R, Scott A, Tien YC, Murray R, Sabourin L, Zhang Y, Topp E. 2013. Impact of manure fertilization on the abundance of antibiotic-resistant bacteria and frequency of detection of antibiotic resistance genes in soil and on vegetables at harvest. Appl Environ Microbiol 79:5701–5709. https://doi.org/10.1128/AEM.01682-13.
17. Larney FJ, Hao X. 2007. A review of composting as a management alternative for beef cattle feedlot manure in southern Alberta, Canada. Bioresour Technol 98:3221–3227. https://doi.org/10.1016/j.biortech.2006.07.005.
18. Martens W, Böhm R. 2009. Overview of the ability of different treatment methods for liquid and solid manure to inactivate pathogens. Bioresour Technol 100:5374–5378. https://doi.org/10.1016/j.biortech.2009.01.014.
19. US Food and Drug Administration. 2020. Produce safety rule. 21 CFR §§ 112.
20. US Environmental Protection Agency. 1994. A plain English guide to the EPA part 503 biosolids rule, EPA/832/R-93/003. US Environmental Protection Agency, Washington, DC.
21. Canadian Council of Ministers of the Environment. 2005. Guideline for compost quality. PN1340. Canadian Council of Ministers of the Environment, Winnipeg, Manitoba, Canada.
22. Batham M, Gupta R, Tiwari A. 2013. Implementation of bulking agents in composting: a review. J Bioremediat Biodegrad 4:205.
23. Solano ML, Iriarte F, Ciria P, Negro MJ. 2001. Performance characteristics of three aeration systems in the composting of sheep manure and straw. J Agric Eng Res 79:317–329. https://doi.org/10.1006/jaer.2001.0703.
24. Sura S, Degenhardt D, Cessna AJ, Larney FJ, Olson AF, McAllister TA. 2014. Dissipation of three veterinary antimicrobials in beef cattle feedlot manure stockpiled over winter. J Environ Qual 43:1061–1070. https://doi.org/10.2134/jeq2013.11.0455.
25. Larney FJ, Buckley KE, Hao X, McCaughey WP. 2006. Fresh, stockpiled, and composted beef cattle feedlot manure: nutrient levels and mass balance estimates in Alberta and Manitoba. J Environ Qual 35:1844–1854. https://doi.org/10.2134/jeq2005.0440.
26. Ezzariai A, Hafidi M, Khadra A, Aemig Q, El Fels L, Barret M, Merlina G, Patureau D, Pinelli E. 2018. Human and veterinary antibiotics during composting of sludge or manure: global perspectives on persistence, degradation, and resistance genes. J Hazard Mater 359:465–481. https://doi.org/10.1016/j.jhazmat.2018.07.092.
27. Liao H, Lu X, Rensing C, Friman VP, Geisen S, Chen Z, Yu Z, Wei Z, Zhou S, Zhu Y. 2018. Hyperthermophilic composting accelerates the removal of antibiotic resistance genes and mobile genetic elements in sewage sludge. Environ Sci Technol 52:266–276. https://doi.org/10.1021/acs.est.7b04483.
28. Sharma R, Larney FJ, Chen J, Yanke LJ, Morrison M, Topp E, McAllister TA, Yu Z. 2009. Selected antimicrobial resistance during composting of manure from cattle administered sub-therapeutic antimicrobials. J Environ Qual 38:567–575. https://doi.org/10.2134/jeq2007.0638.
29. Wang R, Zhang J, Sui Q, Wan H, Tong J, Chen M, Wei Y, Wei D. 2016. Effect of red mud addition on tetracycline and copper resistance genes and microbial community during the full scale swine manure composting. Bioresour Technol 216:1049–1057. https://doi.org/10.1016/j.biortech.2016.06.012.
30. Gou M, Hu H-W, Zhang Y-J, Wang J-T, Hayden H, Tang Y-Q, He J-Z. 2018. Aerobic composting reduces antibiotic resistance genes in cattle manure and the resistome dissemination in agricultural soils. Sci Total Environ 612:1300–1310. https://doi.org/10.1016/j.scitotenv.2017.09.028.
31. Chen Z, Wang Y, Wen Q. 2018. Effects of chlortetracycline on the fate of multi-antibiotic resistance genes and the microbial community during swine manure composting. Environ Pollut 237:977–987. https://doi.org/10.1016/j.envpol.2017.11.009.
32. Qian X, Sun W, Gu J, Wang X, Sun J, Yin Y, Duan ML. 2016. Variable effects of oxytetracycline on antibiotic resistance gene abundance and the bacterial community during aerobic composting of cow manure. J Hazard Mater 315:61–69. https://doi.org/10.1016/j.jhazmat.2016.05.002.
33. Wang J, Ben W, Zhang Y, Yang M, Qiang Z. 2015. Effects of thermophilic composting on oxytetracycline, sulfamethazine, and their corresponding resistance genes in swine manure. Environ Sci Process Impacts 17:1654–1660. https://doi.org/10.1039/c5em00132c.
34. Xie W, Yang X, Li Q, Wu L, Shen Q, Zhao FJ. 2016. Changes in antibiotic concentrations and antibiotic resistome during commercial composting of animal manures. Environ Pollut 219:182–190. https://doi.org/10.1016/j.envpol.2016.10.044.

35. Qian X, Gu J, Sun W, Wang X-J, Su J-Q, Stedfeld R. 2018. Diversity, abundance, and persistence of antibiotic resistance genes in various types of animal manure following industrial composting. J Hazard Mater 344:716–722. https://doi.org/10.1016/j.jhazmat.2017.11.020.
36. Selvam A, Xu D, Zhao Z, Wong JWC. 2012. Fate of tetracycline, sulfonamide and fluroquinolone resistance genes and the changes in bacterial diversity during composting of swine manure. Bioresour Technol 126:383–390. https://doi.org/10.1016/j.biortech.2012.03.045.
37. Xu S, Sura S, Zaheer R, Wang G, Smith A, Cook S, Olson AF, Cessna AJ, Larney FJ, McAllister TA. 2016. Dissipation of antimicrobial resistance determinants in composted and stockpiled beef manure. J Environ Qual 45:528–536. https://doi.org/10.2134/jeq2015.03.0146.
38. Staley ZR, Schmidt AM, Woodbury B, Eskridge KM, Durso L, Li X. 2020. Use of corn residue as a compost bulking agent may contribute antibiotic resistant bacteria to compost. J Environ Qual 49:745–753. https://doi.org/10.1002/jeq2.20017.
39. Parkinson R, Gibbs P, Burchett S, Misselbrook T. 2004. Effect of turning regime and seasonal weather conditions on nitrogen and phosphorus losses during aerobic composting of cattle manure. Bioresour Technol 91:171–178. https://doi.org/10.1016/s0960-8524(03)00174-3.
40. Gonthier A, Guérin-Faublée V, Tilly B, Delignette-Muller M-L. 2001. Optimal growth temperature of O157 and non-O157 *Escherichia coli* strains. Lett Appl Microbiol 33:352–356. https://doi.org/10.1046/j.1472-765x.2001.01010.x.
41. Byappanahalli MN, Nevers MB, Korajkic A, Staley ZR, Harwood VJ. 2012. Enterococci in the environment. Microbiol Mol Biol Rev 76:685–706. https://doi.org/10.1128/MMBR.00023-12.
42. Su J-Q, Wei B, Ou-Yang W-Y, Huang F-Y, Zhao Y, Xu H-J, Zhu Y-G. 2015. Antibiotic resistome and its association with bacterial communities during sewage sludge composting. Environ Sci Technol 49:7356–7363. https://doi.org/10.1021/acs.est.5b01012.
43. Blazy V, Guardia A, Benoist JC, Daumoin M, Lemasle M, Wolbert D, Barrington S. 2014. Odorous gaseous emissions as influence by process condition for the forced aeration composting of pig slaughterhouse sludge. Waste Manag 34:1125–1138. https://doi.org/10.1016/j.wasman.2014.03.012.
44. Zhang L, Sun X. 2016. Influence of bulking agents on physical, chemical, and microbiological properties during the two-stage composting of green waste. Waste Manag 48:115–126. https://doi.org/10.1016/j.wasman.2015.11.032.
45. Wang C, Dong D, Strong PJ, Zhu W, Ma Z, Qin Y, Wu W. 2017. Microbial phylogeny determines transcriptional response of resistome to dynamic composting processes. Microbiome 5:103. https://doi.org/10.1186/s40168-017-0324-0.
46. Liao H, Friman V-P, Geisen S, Zhao Q, Cui P, Lu X, Chen Z, Yu Z, Shungui Z. 2019. Horizontal gene transfer and shifts in linked bacterial community composition are associated with maintenance of antibiotic resistance genes during food waste composting. Sci Total Environ 660:841–850. https://doi.org/10.1016/j.scitotenv.2018.12.353.
47. NRAES. 1999. Field guide to on-farm composting. Natural Resource, Agriculture, and Engineering Service Cooperative Extension, Ithaca, NY.
48. Agga GE, Arthur TM, Durso LM, Harhay DM, Schmidt JW. 2015. Antimicrobial-resistant bacterial populations and antimicrobial resistance genes obtained from environments impacted by livestock and municipal waste. PLoS One 10:e0132586. https://doi.org/10.1371/journal.pone.0132586.
49. Schmidt JW, Agga GE, Bosilevac JM, Brichta-Harhay DM, Shackelfor SD, Wang R, Wheeler TL, Arthur TM. 2015. Occurrence of antimicrobial-resistant *Escherichia coli* and *Salmonella enterica* in the beef cattle production and processing continuum. Appl Environ Microbiol 81:713–725. https://doi.org/10.1128/AEM.03079-14.
50. Vikram A, Rovira P, Agga GE, Arthur TM, Bosilevac JM, Wheeler TL, Morley PS, Belk KE, Schmidt JW. 2017. Impact of "raised without antibiotics" beef cattle production practices on occurrences of antimicrobial resistance. Appl Environ Microbiol 83:e01682-17. https://doi.org/10.1128/AEM.01682-17.
51. Agga GE, Schmidt JW, Arthur TM. 2016. Effect of in-feed chlortetracycline prophylaxis in beef cattle on animal health and antimicrobial-resistant *Escherichia coli*. Appl Environ Microbiol 82:7197–7204. https://doi.org/10.1128/AEM.01928-16.
52. FDA. 2013. 2012 retail meat report. National Antimicrobial Resistance Monitoring System, US Department of Health and Human Services, US Food and Drug Administration, Rockville, MD.
53. US Environmental Protection Agency. 2017. Analytical methods approved for compliance monitoring under the revised total coliform rule. EPA 821-F-17-004.
54. Nocker A, Cheung C-Y, Camper AK. 2006. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J Microbiol Methods 67:310–320. https://doi.org/10.1016/j.mimet.2006.04.015.
55. Nocker A, Sossa-Fernandez P, Burr MD, Camper AK. 2007. Use of propidium monoazide for live/dead distinction in microbial ecology. Appl Environ Microbiol 73:5111–5117. https://doi.org/10.1128/AEM.02987-06.
56. Suzuki MT, Taylor LT, DeLong EF. 2000. Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5′-nuclease assays. Appl Environ Microbiol 66:4605–4614. https://doi.org/10.1128/AEM.66.11.4605-4614.2000.
57. Rosewarne CP, Pettigrove V, Stokes HW, Parsons YM. 2010. Class 1 integrons in benthic bacterial communities: abundance, association with TN*402*-like transportation modules and evidence for coselection with heavy-metal resistance. FEMS Microbiol Ecol 72:35–46. https://doi.org/10.1111/j.1574-6941.2009.00823.x.
58. Koike S, Aminov RI, Yannarell AC, Gans HD, Krapac IG, Chee-Sanford JC, Mackie RI. 2010. Molecular ecology of macrolide-lincosamide-streptogramin B methylases in waste lagoons and subsurface waters associated with swine production. Microb Ecol 59:487–498. https://doi.org/10.1007/s00248-009-9610-0.
59. Aminov RI, Garrigues-Jeanjean N, Mackie RI. 2001. Molecular ecology of tetracycline resistance: development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. Appl Environ Microbiol 67:22–32. https://doi.org/10.1128/AEM.67.1.22-32.2001.
60. Aminov RI, Chee-Sanford JC, Garrigues N, Teferedegne B, Krapac IJ, White BA, Mackie RI. 2002. Development, validation, and application of PCR primers for detection of tetracycline efflux genes of gram-negative bacteria. Appl Environ Microbiol 68:1786–1793. https://doi.org/10.1128/AEM.68.4.1786-1793.2002.
61. Storteboom H, Arabi M, Davis JG, Crimi B, Pruden A. 2010. Identification of antibiotic-resistance-gene molecular signatures suitable as tracers of pristine river, urban, and agricultural sources. Environ Sci Technol 44:1947–1953. https://doi.org/10.1021/es902893f.
62. Peak N, Knapp CW, Yang RK, Hanfelt MM, Smith MS, Aga DS, Graham DW. 2007. Abundance of six tetracycline resistance genes in wastewater lagoons at cattle feedlots with different antibiotic use strategies. Environ Microbiol 9:143–151. https://doi.org/10.1111/j.1462-2920.2006.01123.x.