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Antibiotic resistance genes in swine manure slurry as affected by pit additives and facility disinfectants

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Antibiotic resistance genes in swine manure slurry as affected by pit additives and facility disinfectants

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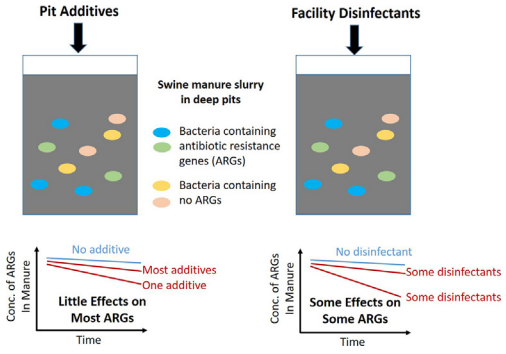
HIGHLIGHTS

- Effects of chemicals on ARGs in swine manure storage facilities were studied.
- Most but one pit additives had little impacts on the ARGs tested.
- Some facility disinfectants significantly reduced the levels of some ARGs.

GRAPHICAL ABSTRACT



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ABSTRACT

Manure storage facilities are critical control points to reduce antibiotic resistance genes (ARGs) in swine manure slurry before the slurry is land applied. However, little is known about how exogenous chemicals entering the manure storage facilities may affect the fate of ARGs. The objective of this study was to analyze the impact of six commonly used pit additives and four facility disinfectants on the concentration of ARGs in swine manure slurry. Bench scale reactors, each containing approximately 50 L of liquid swine manure, were dosed with additives or disinfectants and were sampled for 40 days. Seven antibiotic resistance genes along with the *intI1* gene and the 16S rRNA gene were monitored. Out of the six additives tested, Sludge Away significantly reduced the time-averaged absolute abundance of *erm*(C), *erm*(F), *tet*(Q), and the 16S rRNA gene as compared to the no additive control. Out of the four disinfectants tested, Tek-Trol significantly reduced the time-averaged absolute abundance of *erm*(B), *erm*(C), *erm*(F), *intI1*, *tet*(Q), and *tet*(X) than did the no-disinfectant control. According to Spearman's rank correlation, three genes *erm*(F), *tet*(Q), and *tet*(X) showed a strong to perfectly positive correlation and the two genes *erm*(B) and *tet*(O) showed a moderate to strong correlation in both the additive and disinfectant tests. Overall, the disinfectants were more effective in controlling the absolute abundance of ARGs than were the pit additives.

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Table 1
Information about the pit additives used in simulated storage.

Pit additive	Manufacturer	Purpose	Active ingredient	Dose into 50 liter slurry
Coban® 90	Elanco	Pit foaming	Monensin	0.295 g
Manure Magic®	Drylet	Crusting, odor control, pit foaming, solids reduction	Patent particles, mixed microbial cultures	0.295 g
MOC-7	Ag odor control	Odor control, solids reduction, anti-crusting	N/A	1.7 mL
More Than Manure®	Verdesian life sciences	Air quality preservation, nutrient preservation, solids reduction	Maleic-itaconic copolymer with ammonium and calcium salts	3.3 mL
Sludge away	Microbe-lift	Solids reduction, odor control	Humic and fulvic acids, purple sulfur bacteria	9.8 mL
Sulfi-Doxx	Direct biologicals	Odor control, foaming control, solids reduction, promote microbe growth	Humic and fulvic acids, mixed microbial cultures	0.82 mL

1. Introduction

Antibiotic resistance could threaten the effectiveness of antibiotics against bacterial infections in humans and livestock. Research shows the linkage between the antibiotic resistance developed in livestock facilities to antibiotic resistant infections in humans (Tang et al., 2017). In livestock facilities, antibiotics are administered to livestock to treat and prevent diseases (C.-S. et al., 2009; Dibner and Richards, 2005). Antibiotics chlortetracycline, lincomycin, and tiamulin, which belong to the antibiotic classes of tetracycline, lincosamides, and pleuromutilins, are used in swine production (Duerschner et al., 2020) and their residues may occur at concentrations ranging from sub mg to several mg per kg wet weight of manure solids prior to land application (Hall et al., 2020; Joy et al., 2013). Under the selective pressure of antibiotics, resistance develops in the guts of the animals and antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) are excreted along with antibiotic residues in wastes. During manure storage some ARB and ARGs tend to persist (Joy et al., 2014; Schlüsener et al., 2006). Finally, through land application, ARB and ARGs originating in livestock wastes may enter the environment (Hall et al., 2020), temporarily increasing the abundance and diversity of ARGs in the receiving environments.

Swine manure slurry is often collected in indoor storage pits, in enclosed storage tanks, or treatment lagoons outside swine barns (Chastain and Henry, 2002). Manure slurry is stored in these facilities until it is applied on croplands as a fertilizer. While in these locations, swine manure slurry undergoes both aerobic and anaerobic processes (Key et al., 2011). Aerobic processes take place in the top layer of swine manure slurry where oxygen can diffuse into the liquid. Beneath the surface, anaerobic processes occur where organic materials are converted into hydrogen sulfide, ammonia, and methane (Chastain and Henry, 2002; Ni et al., 2008). Exogenous compounds that enter manure storage containments can affect the aerobic and anaerobic microbial processes. For example, chlortetracycline has been shown to cause a 28% reduction in the generation of methane and carbon dioxide (Stone et al., 2009). Previous studies using full-scale and lab-scale systems have reported both increases and decreases in the absolute and relative abundance of ARGs during manure storage (Joy et al., 2014; Chen et al., 2010; Wang et al., 2012), and the trends can be related to the antibiotic residues and other added chemicals.

Swine facility operators often add additives to storage pits for multiple purposes: reducing solids, lowering methane gas production, reducing ammonia volatilization, controlling foaming, and killing pathogens. For example, Manure Magic® is an additive that has been advertised to reduce methane production, control foaming, and increase solids destruction (Polson and Andersen, 2017). Coban® 90 is an additive containing monensin, an antibiotic used only in agriculture, as the active ingredient. By inhibiting the microbes producing acetic acid, an important precursor for methane generation, monensin can reduce the generation of biogas-containing foam during swine waste storage (Clanton et al., 2012), thus reducing the risk of explosion from the mixing of biogas and air in the presence of a spark from a heater, motor, welding, or grinding equipment (Clanton et al., 2012). Another commonly used additive is Sulfi-Doxx, which is marketed to enhance microbial growth and thus increase solids destruction.

Disinfectants are used to sanitize surfaces inside swine production facilities. Regular flushing of the slatted floor is also a common practice. Following disinfection, water is used to rinse off disinfectant residues and flushes them into manure storage containments (e.g., pits, tanks, or lagoons). Different disinfectants have varying effects on the microbes in the storage containments and resistance against a certain disinfectant may arise after prolonged exposure (Thompson et al., n.d.). For example, bacteria facing long-term exposure to low-levels of chlorine has been shown to increase their tolerance to this oxidative disinfectant (Ridgway and Olson, 1982).

Previous studies reported how ARG concentrations change during the storage of livestock wastes (Joy et al., 2014; Koike et al., 2007; McKinney et al., 2010; Pei et al., 2007; Zhang et al., 2013a). However, little is known about how exogenous compounds, such as pit additives and facility disinfectants, may affect the concentrations of ARGs in swine manure slurry during storage. The objective of this study was to analyze the impact of six commonly used pit additives and four facility disinfectants on the concentration of ARGs in swine manure slurry. Swine manure storage pits were simulated using 57-L stainless steel tanks. Following the addition of pit additives or facility disinfectants, multiple ARGs were monitored over a 40-day storage period. Statistical analyses were conducted to evaluate the significance of the trends observed among additives and disinfectants. Consequently, the results from this study may aid swine producers in choosing compounds that can reduce ARGs in swine manure slurry during storage.

2. Materials and methods

2.1. Manure collection and characterization

Swine manure slurry was collected from a swine production facility in southeast Nebraska in January and March 2018 for the testing of pit additives and facility disinfectants, respectively. Slurry was taken from storage pits through the ventilation entrance and shipped back to the University of Nebraska – Lincoln (UNL). The solid content of the two batches of manure slurry was averaged at 5.4% and 7.9% for the additive and the disinfectant experiments, respectively.

2.2. Manure storage experiments

Each simulated storage reactor, a 57-L stainless steel pot placed in a greenhouse at UNL, received 50 L of well mixed manure slurry. In January 2018, manure in simulated storage reactors was dosed with the following pit additives: Manure Magic® (DryLet), MOC 7 (Ag Odor Control), More Than Manure® (Verdesian), Coban® 90 (Elanco), Sulfi-Doxx (Direct Biologicals), and Sludge Away (Ecological Laboratories) (Table 1). Manure in simulated storage reactors was dosed in March 2018 with the following facility disinfectants: Clorox® (the Clorox

Table 2
Information of the facility disinfectants used in simulated storage.

Disinfectant	Type	Active ingredient	Recommended rinse	Dose
Clorox®	Halogen	Sodium hypochlorite	Yes	180 mL
Pi Quat	QAC	Alkyl dimethyl benzyl ammonium chloride, alkyl dimethyl ethylbenzyl ammonium chloride	No	360 mL
Tek-Trol	Phenol	Para-tertiary-amylphenol, ortho-benzyl-para-chlorophenol, ortho-phenylphenol	Yes	180 mL
Virkon™	Oxidizer	Potassium peroxymonosulfate, sodium chloride, other	No	360 mL

Company), Pi Quat (Preserve International), Tek-Trol (Bio-Tek Industries, Inc.), and Virkon™ (Antec International Limited) (Table 2).

For the additive experiment, each additive was added to two randomly assigned reactors and was dosed according to manufacturer guidelines (Table 1). Two reactors were designated as control reactors and received no additive. For the disinfectant experiment, each disinfectant was also added to two randomly assigned reactors and dosed according to common practices: the volume of disinfectants used was calculated by multiplying the area by 2.5 to account for walls and other protruding elements in a swine facility and then by 0.03 cm to ensure full saturation of the area. If a rinse was recommended by the manufacturer (i.e., Clorox® and Tek-Trol), then the treatment dose was cut by half and an equal volume of DI water was added to simulate the rinse water. The volumes of the disinfectants used per reactor are listed in Table 2.

Reactors were placed on tables in the greenhouse, covered from direct sunlight, and lids were left off to simulate open storage. Samples were taken prior to the addition of pit additives or disinfectants and then again 1, 2, 5, 10, 14, 21, 32, and 40 days after the addition. Before sampling, the pH and dissolved oxygen (DO) of each pot were recorded using a Thermo-Fisher Orion Star Water Quality Meter. The probes were rinsed with DI water between reactors to avoid cross contamination. Thereafter a paint mixer, one designated paint mixer per set of duplicate reactors, was used to homogenize the manure. Samples were scooped into labeled containers and transported to the laboratory. At the laboratory, each sample was mixed in a blender and an aliquot was transferred into containers, which were stored at −20 °C until analyses. The temperature of the solution inside the reactors ranged between 12.5 °C–17.9 °C between 1/25 to 3/6 for the additive experiment and 11.9 °C–18.9 °C between 3/30 and 5/10/2018 for the disinfectant experiment.

2.3. ARG analysis

Frozen manure slurry samples were thawed at 4 °C. Samples were thoroughly vortexed before being loaded into MagMAX bead tubes using wide bore filter pipette tips. The samples were extracted using the MagMAX™ Total Nucleic Acid Isolation Kit (Applied Biosystems™) on a Kingfisher Flex (ThermoFisher Scientific) as liquid samples. Extracted samples were purified with OneStep™ PCR Inhibitor Removal Kit (Zymo Research). Synthesized gBlocks fragments (Integrated DNA Technologies) were used to make qPCR standards. The qPCR reactions were performed on a Mastercycler ep realplex 2 thermocycler (Eppendorf) using KiCqStart® SYBR® Green qPCR ReadyMix™ (Sigma Aldrich). Assay setup and cycling conditions were adopted from published studies with some optimization modifications. Primer sequences, reaction conditions, reaction efficiencies, and references are listed in Tables S1 and S2. Linear ranges and reaction efficiencies are reported in Table S3.

2.4. Statistical analysis

Statistical analyses were conducted using SAS and R (The R Foundation for Statistical Computing). ANOVA in SAS was used for analyzing the impact of treatment on the $\log_{10}$-transformed gene concentrations. If a treatment had significant impacts according to ANOVA, Fisher's protected least significant difference (LSD) was used to compare treatment means at $p < 0.05$. To evaluate the changes in $\log_{10}$-transformed gene concentration over time, growth curve analysis was performed as described in Eskridge and Stevens (1987). Student's *t*-tests were also performed on individual treatments to determine if ARG concentrations significantly increased or decreased over time. Gene correlation analysis was conducted in R with Spearman's rank correlations where correlations were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Pit additives

Evaporation resulted in loss of slurry volume over the 40-day period. To account for the volume loss due to evaporation, sodium iron (Na^+) in the manure slurry was treated as a conservative tracer. All of the gene concentrations reported in the paper were adjusted for volume loss due to evaporation. ARGs belonging to the *tet* and *erm* families were focused in this study because chlortetracycline and lincomycin were the two antibiotics that were used routinely at the swine facility. Endpoint PCR on initial manure samples were used to determine the ARGs to be included in subsequent qPCR analysis. Based on endpoint PCR results, *erm*(B), *erm*(C), *erm*(F), *intI1*, *tet*(G), *tet*(O), *tet*(Q), and *tet*(X) were chosen for qPCR analyses.

The absolute abundances of *erm*(B), *erm*(C), *erm*(F), *intI1*, *tet*(G), *tet*(O), *tet*(Q), *tet*(X), and the 16S rRNA gene under the treatment of various pit additives, over a 40-day storage period are plotted in Fig. 1. For most of the genes tested, the six pit additives exhibited trends similar to each other and to the control. One exception was Sludge Away, which exhibited a noticeably different trend from the rest of the test conditions for *erm*(C), *erm*(F), *tet*(Q), and the 16S rRNA gene.

The data were further analyzed using statistical methods to answer two questions. The first question was whether the addition of a pit additive had any impact on the persistence of genes during manure storage (i.e., comparison in gene abundance between treatment and control reactors). The second question was whether longer storage may lead to lower gene abundance in manure (i.e., comparison in gene abundance between later time points and time zero).

To answer the first question, the absolute abundance of each gene was averaged over the 40-day period and reported in Table 3. ANOVA analyses showed that the addition of pit additives had significant impacts on the averaged gene abundance for all the genes tested ($p < 0.05$, Table 3). LSD tests showed that three out of the six pit additives (i.e., MOC-7, More Than Manure®, and Sulfi-Doxx) resulted in significantly higher average absolute abundances of at least eight out of nine genes when compared to the no additive controls. Similarly, Coban® 90 and Manure Magic® significantly increased the absolute abundance of a subset of the genes tested. Noticeably, Sludge Away significantly reduced the average absolute abundance of *erm*(C), *erm*(F), *tet*(Q), and the 16S rRNA gene as compared to the control (Table 3).

To answer the second question, the temporal trend of the absolute abundance of each gene was analyzed for individual pit additives. Growth curve analysis (Eskridge and Stevens, 1987) was fit with cubic trends over time, which revealed virtually no differences among additive treatments and controls. However, some time trend effects were significant after averaging over all additives and control where the changes in the absolute abundance of genes were gene specific: the absolute abundance of *erm*(F) and *tet*(Q) averaged over all additives and control increased over time ($p < 0.05$).

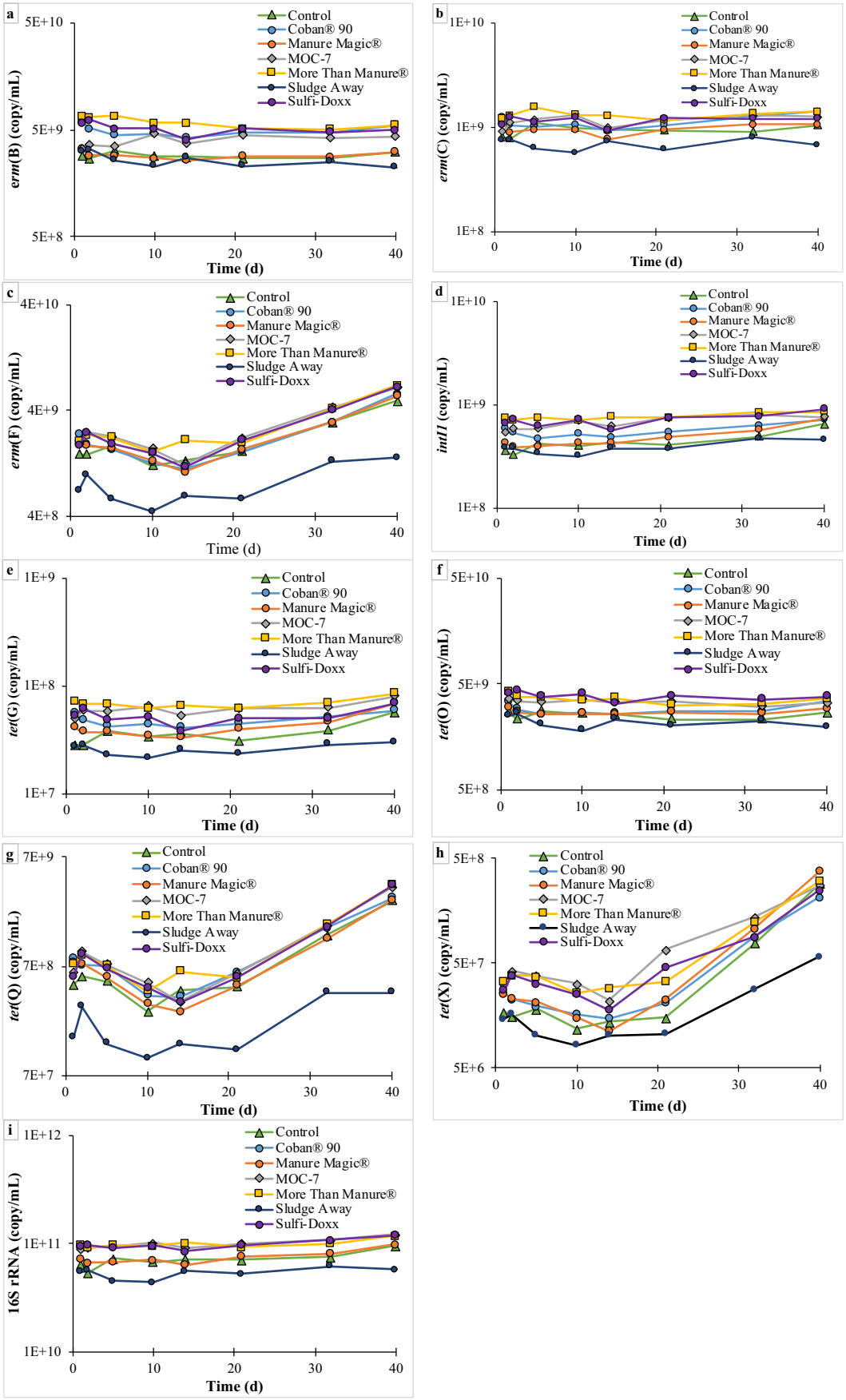

Fig. 1. Impact of pit additives on the absolute abundance of (a) *erm*(B), (b) *erm*(C), (c) *erm*(F), (d) *intI1*, (e) *tet*(G), (f) *tet*(O), (g) *tet*(Q), (h) *tet*(X), and (i) the 16S rRNA gene during simulated storage. For the visibility of the figure, error bars representing the ranges from duplicate reactors were not included.

Table 3
Time-averages of the absolute abundance of genes in manure over the 40-day simulated storage.[a] Red and green backgrounds indicate significantly higher and lower, respectively, average abundance than that of the control.

	16S rRNA	*erm*(B)	*erm*(C)	*erm*(F)	*intI1*	*tet*(G)	*tet*(O)	*tet*(Q)	*tet*(X)
Pit additive[a]	(copy mL^{-1})	(copy mL^{-1})	(copy mL^{-1})	(copy mL^{-1})	(copy mL^{-1})	(copy mL^{-1})	(copy mL^{-1})	(copy mL^{-1})	(copy mL^{-1})
Control	7.0×10^{10} b	2.8×10^{9} c	9.3×10^{8} b	1.9×10^{9} c	4.2×10^{8} d	3.5×10^{7} de	2.5×10^{9} cd	6.2×10^{8} c	2.6×10^{7} d
Coban® 90	8.0×10^{10} ab	4.8×10^{9} ab	1.1×10^{9} ab	2.0×10^{9} b	5.5×10^{8} bc	4.6×10^{7} bd	2.8×10^{9} bc	7.5×10^{8} ab	3.0×10^{7} b
Manure Magic®	7.4×10^{10} b	2.9×10^{9} c	9.6×10^{8} b	2.0×10^{9} b	4.7×10^{8} cd	4.1×10^{7} d	2.7×10^{9} c	6.6×10^{8} b	3.4×10^{7} ab
MOC-7	1.0×10^{11} a	3.9×10^{9} b	1.1×10^{9} ab	2.4×10^{9} ab	6.6×10^{8} ab	6.0×10^{7} ab	3.3×10^{9} ab	8.2×10^{8} ab	5.5×10^{7} a
MTM[b]	9.9×10^{10} a	5.8×10^{9} a	1.3×10^{9} a	2.5×10^{9} a	7.6×10^{8} a	6.8×10^{7} a	3.5×10^{9} a	8.8×10^{8} a	5.0×10^{7} a
Sludge Away	5.3×10^{10} c	2.6×10^{9} c	6.9×10^{8} c	7.6×10^{8} d	3.8×10^{8} d	2.5×10^{7} e	2.2×10^{9} d	1.9×10^{8} d	1.6×10^{7} d
Sulfi-Doxx	9.8×10^{10} a	5.0×10^{9} a	1.1×10^{9} ab	2.3×10^{9} ab	7.1×10^{8} a	5.1×10^{7} ab	3.8×10^{9} a	7.6×10^{8} ab	4.3×10^{7} ab
p-value	0.004	<0.001	0.003	<0.001	0.001	0.005	0.004	<0.001	0.007
Facility disinfectant									
Control	9.4×10^{10}	9.4×10^{9} a	1.1×10^{9} a	3.2×10^{9} a	3.7×10^{8} a	1.5×10^{7}	7.4×10^{9}	1.2×10^{9} ab	3.3×10^{7} a
Clorox®	7.1×10^{10}	6.0×10^{9} ab	8.8×10^{8} b	4.1×10^{9} a	2.1×10^{8} b	1.4×10^{7}	4.2×10^{9}	1.9×10^{9} a	4.8×10^{7} a
Pi Quat	9.0×10^{10}	8.8×10^{9} a	1.2×10^{9} a	1.4×10^{9} b	2.8×10^{8} ab	1.3×10^{7}	6.8×10^{9}	3.9×10^{8} bc	1.7×10^{7} b
Tek-Trol	5.4×10^{10}	3.9×10^{9} b	9.2×10^{8} b	1.4×10^{9} b	2.0×10^{8} b	1.1×10^{7}	4.1×10^{9}	3.0×10^{8} c	9.3×10^{6} c
Virkon™	1.0×10^{11}	4.5×10^{9} b	9.3×10^{8} b	4.1×10^{9} a	3.5×10^{8} a	1.9×10^{7}	4.9×10^{9}	1.4×10^{9} a	4.7×10^{7} a
p-value	0.184	0.021	0.009	0.016	0.036	0.286	0.063	0.026	0.002

[b]MTM, More Than Manure®.

[a] Values reported under "Pit Additive" and "Facility Disinfectant" are averages over 40-day storage (i.e., average of values from Day 1, 2, 5, 10, 14, 21, 32, 40). The *p*-values show if the average abundance of individual genes is significantly affected by the use of additives or disinfectants. If yes, then the average abundances are labeled with letters based on LSD tests at the $p < 0.05$ level.

The largest impact on absolute abundance was seen in the Sludge Away treatment. Sludge Away was the only pit additive that resulted in lower ARG abundance than the control reactors (i.e., *erm*(C), *erm*(F) and *tet*(Q)). Sludge Away contains humic and fulvic acids along with purple bacteria (Table 1). The addition of purple sulfur bacteria may partially account for the phenomenon by outcompeting ARG-carrying bacteria (Saikaly and Oerther, 2004). Knowledge on how humic and fulvic acids affect bacteria in manure is lacking. One study claimed that humic and fulvic acids could benefit soil bacteria by inducing metabolic changes that allow cells to utilize a wider range of substrates (Visser, 1985).

Sulfi-Doxx and Manure Magic® also contain microbes, with the former containing humic and fulvic acids as well. Both additives resulted in elevated time-averaged abundance of genes as compared to the control (Table 3). This suggests that the composition (and abundance, which is unknown from labels) of the microbes added may play a role in affecting ARG-carrying bacteria during manure storage. MOC-7 and More Than Manure® significantly increased the time-average concentration of at least eight out of the nine genes tested. MOC-7's active ingredients were not disclosed by the manufacturer, while More Than Manure® was reported to contain maleic-itaconic polymers along with ammonium and calcium salts (Table 1). Maleic-itaconic polymers are urease inhibitors, which can reduce the conversion of urea to ammonia to minimize volatilization (Chien et al., 2014; Parker et al., 2005). High concentrations of ammonia can lower the specific growth rates of microbes (Hansen et al., 1998). Therefore, the increase in absolute gene abundance following More Than Manure® treatment could be attributed to a reduced ammonia concentration in the swine slurry. Compared to the control, Coban® 90 resulted in higher abundance for three ARGs and *intI1*. The active ingredient in Coban® 90 is the antibiotic monensin. Monensin is an ionophore antimicrobial that has been related to the presence of resistance genes in both urban and agricultural environments (Pei et al., 2006).

3.2. Facility disinfectants

The absolute abundances of *erm*(B), *erm*(C), *erm*(F), *intI1*, *tet*(G), *tet*(O), *tet*(Q), and *tet*(X), under the treatment of various facility disinfectants, over 40-day simulated storage are plotted in Fig. 2. These raw data were further processed in the same manner as those used in the pit additive tests.

The absolute abundance of each gene was averaged over the 40-day period and reported for each disinfectant in Table 3. Compared to the controls, where no disinfectant was added, Tek-Trol significantly reduced the averaged absolute abundance of *erm*(B), *erm*(C), *erm*(F), *intI1*, *tet*(Q), and *tet*(X) in manure slurry. Pi Quat significantly decreased the absolute abundance of *erm*(F) and *tet*(X). Clorox® caused a significant decrease in *erm*(C) and *intI1*, and Virkon™ addition led to a significant decrease in *erm*(B) and *erm*(C) (Table 3).

Similarly to pit additives, the temporal trend of the absolute abundance of each gene was modeled using cubic growth curve analysis (Eskridge and Stevens, 1987). The absolute abundance of *tet*

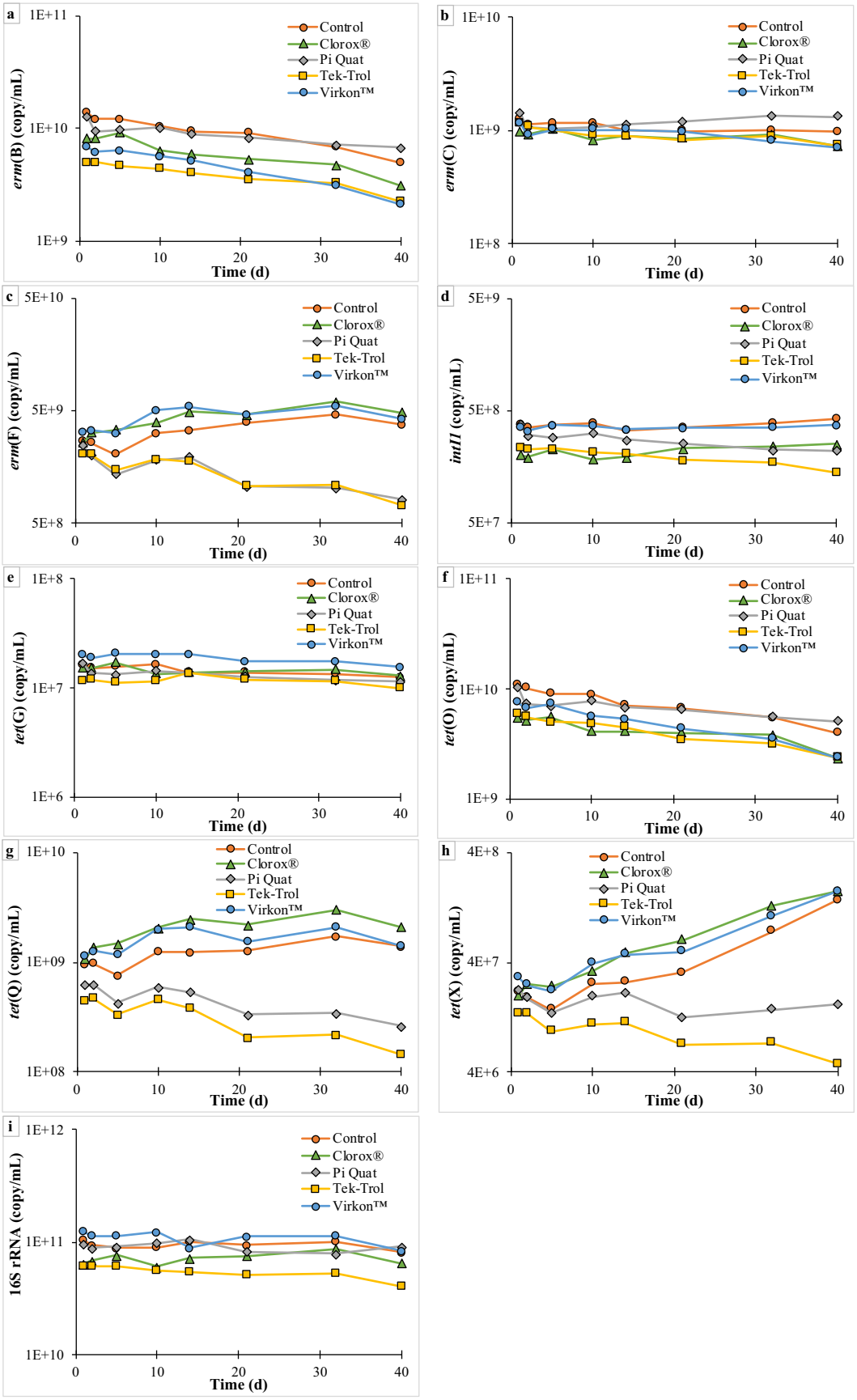

Fig. 2. Impact of facility disinfectants on the absolute abundance of (a) *erm*(B), (b) *erm*(C), (c) *erm*(F), (d) *intI1*, (e) *tet*(G), (f) *tet*(O), (g) *tet*(Q), (h) *tet*(X), and (i) the 16S rRNA gene during simulated storage. For the visibility of the figure, error bars representing the ranges from duplicate reactors were not included.

(X) averaged over all disinfectants and control decreased over time ($p < 0.05$). In addition, two of the disinfectants, Pi Quat and Tek-Trol, exhibited significantly lower slopes than the control for *tet*(X).

The disinfectants were overall more effective in controlling the absolute abundance of ARGs than were the pit additives. Out of the four disinfectants tested, Tek-Trol was the most effective disinfectant in reducing the absolute abundance of the genes tested as compared to the control. Tek-Trol is a phenol disinfectant and kills bacterial cells by inducing leakage of intracellular constituents (Chapman, 2003). No specific resistance mechanism has been associated with phenol disinfectants.

Pi Quat is a quaternary ammonium compound (QAC), which has been associated with the occurrence of efflux pump coding gene *qacA* on multidrug resistance plasmids (Chapman, 2003; Sidhu et al., 2002; Tennent et al., 1989). QAC's mode of action is to disrupt the cell membrane and cause lysis (Ioannou et al., 2007). For *erm*(C), which codes for a ribosomal protection protein, Pi Quat was the only disinfectant that did not decrease its absolute abundance as compared to the control (Table 3). The ARG *qacA* was not monitored in this study, however, co-occurrence of *erm*(C) and *qacA* has been observed previously in clinical isolates (Kitti et al., 2018).

The active ingredients in Clorox® and Virkon™ are the oxidizing agents sodium hypochlorite (i.e., free chlorine) and potassium peroxymonosulfate, respectively. Free chlorine can diffuse into bacterial cells and oxidize various cellular structures (Fukuzaki, 2006; Du et al., 2015). Exposure to free chlorine not only increases cells' tolerance to chlorine (Shi et al., 2013), but may also select for antibiotic resistance (Huang et al., 2011). The effects of chlorine on horizontal gene transfer of ARGs can be complicated: chlorine may promote ARG transfer by improving cell permeability (Guo et al., 2015), but it may also lower conjugation frequency by causing a decreased expression of proteins involved in conjugation (Lin et al., 2016). The decrease in certain ARG concentrations by Chlorox and Virkon™ treatment as compared to the control may be caused by oxidation of free-floating DNA in swine slurry (Zhang et al., 2019), which would otherwise be taken up by cells through transformation (Zhang et al., 2013b). Overall, it appeared that lysing agents were more effective in reducing ARGs than oxidizing agents.

ANOVA was conducted on relative abundance of ARGs (normalized to the 16S rRNA gene). Results show that some chemicals resulted in significant increase or decrease in the relative abundance of certain ARGs (Table S4). Compared to the ARG and chemical combinations that exhibited significant difference from the control in the ANOVA results based on absolute abundance (i.e., the shaded cells in Table 3), those in the ANOVA results based on relative abundance (i.e., the shaded cells in Table S4) are much less, suggesting that the chemicals did not selectively inactivate ARG hosts in swine manure slurry.

3.3. Associations of ARGs in simulated storage

The Spearman's rank correlations were used to test associations among the genes (Fig. 3). The abundance for *erm*(F), *tet*(Q), and *tet*(X) demonstrated moderate to perfect positive associations under pit additive treatments and exhibited nearly perfect positive associations under disinfectant treatments. One metagenomic study reported co-occurrence of *erm*(F) and *tet*(Q) (Li et al., 2015). The strong correlation between these two genes and *tet*(X) may be a result of shared bacterial hosts. In addition, there are weak to moderate positive correlations among *erm*(B), *erm*(C), and *tet*(O) during both additive and disinfectant treatments. The strong correlation between *erm*(B) and *tet*(O) has also been reported previously (Li et al., 2015). Finally, the correlations between *intI1* and individual ARGs were investigated and none of the chemicals tested resulted in uniform strengthening or weakening of the correlations (Table S5).

The gene *tet*(X) was one of the ARGs that had the lowest abundance in the swine slurry initially but had the most significant increase over the 40-day period. The gene *tet*(X) codes for an oxygen-dependent tetracycline degrading enzyme initially detected in an obligate anaerobic species (Chopra and Roberts, 2001; Yang et al., 2004). More recent research has shown that it also exists in aerobes (Ghosh et al., 2009). The rapid increase in *tet*(X) in this study could be due to fast growth of the bacterial hosts. In another study simulating anaerobic storage of swine manure slurry, no increase in *tet*(X) was observed (Joy et al., 2014), suggesting that the increase of this gene in the current study might be attributed to the growth of *tet*(X)-carrying aerobic bacteria living close to the surface of the open tank. Finally, no significant correlations ($p > 0.05$) were observed among the three *erm* genes or among the four *tet* genes. This confirms that the mechanisms involved in the spread and increase of resistance genes does not merely rely on the presence of certain antibiotics (Fahrenfeld et al., 2014).

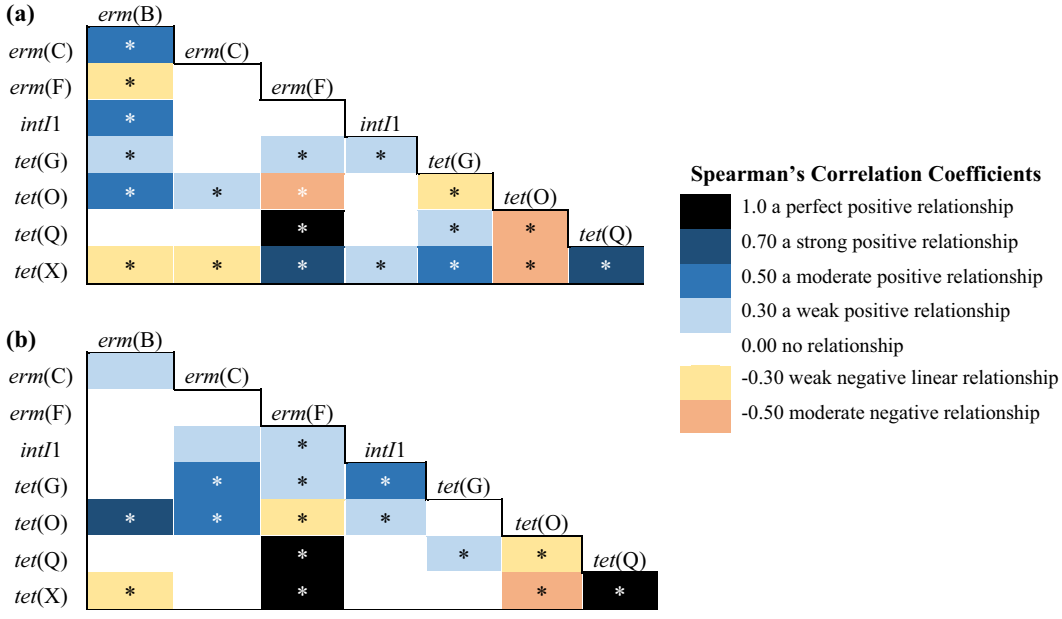

Fig. 3. Heat map for correlation of genes during (a) pit additive treatment and (b) facility disinfectant treatment according to Spearman's rank correlation coefficient. Asterisks (*) indicate the significant correlations ($p < 0.05$).

4. Conclusions

While the fate and transport of ARGs in the environment following the land application of swine manure have been extensively investigated, little is known about how storage conditions affect the ARGs in swine manure slurry. This study was designed to study the effects of two classes of commonly used chemicals on the concentrations of ARGs in simulated deep pits for swine manure storage. Among the six pit additives tested, most of them had little to no effects on the levels of ARGs tested, with the exception of Sludge Away, which significantly reduced the time-averaged absolute abundance of *erm*(C), *erm*(F), *tet*(Q), and the 16S rRNA gene compared to the no-additive control. In comparison, disinfects exhibited stronger inhibitory effects on ARGs. Particularly, Tek-Trol significantly reduced the time-averaged absolute abundance of *erm*(B), *erm*(C), *erm*(F), *intI1*, *tet*(Q), and *tet*(X) than did the no-disinfectant control. Our findings can provide producers with practical guidelines on choosing pit additives and facility disinfectants when ARGs are target pollutants.

CRediT authorship contribution statement

Maria C. Hall: Investigation, Data curation, Visualization, Writing - original draft. **Jon Duerschner:** Investigation, Methodology. **John E. Gilley:** Conceptualization, Methodology, Resources, Writing - review & editing. **Amy M. Schmidt:** Conceptualization, Methodology, Writing - review & editing. **Shannon L. Bartelt-Hunt:** Conceptualization, Methodology, Funding acquisition. **Daniel D. Snow:** Conceptualization, Funding acquisition. **Kent M. Eskridge:** Formal analysis, Writing - review & editing. **Xu Li:** Conceptualization, Methodology, Writing - review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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