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Abstract

Quantifying the fate of antibiotics and antibiotic resistance genes (ARGs) in response to physicochemical factors during storage of manure slurries will aid in efforts to reduce the spread of resistance when manure is land-applied. The objectives of this study were to determine the effects of temperature (10, 35, and 55ºC) and initial pH (5, 7, 9, and 12) on the removal of pirlimycin and prevalence of ARGs during storage of dairy manure slurries. We collected and homogenized feces and urine from five lactating dairy cows treated with pirlimycin and prepared slurries by mixing manure and sterile water. Aliquots (200 ml) of slurry were transferred and incubated in 400 mL glass beakers under different temperatures (10, 35, and 55ºC) or initial pH (5, 7, 9, and 12). Pirlimycin concentration and abundances of 16S rRNA, *mef*A, *tet*(W), and *cfx*A as indicators of total bacteria and ARGs corresponding to macrolide, tetracycline, and β-lactam resistance, respectively, were analyzed during manure incubation. The thermophilic environment (55ºC) increased the deconjugation and removal of pirlimycin, while the acidic shock at pH 5 increased deconjugation but inhibited removal of pirlimycin, suggesting that the chemical stability of pirlimycin could be affected by temperature and pH. The thermophilic environment decreased *mef*A relative abundance on day 7 and 28 (P = 0.02 and 0.04), which indicates the bacteria that encoded *mef*A gene were not thermotolerant. Although *mef*A relative abundance was greater at the pH 9 shock than the rest of pH treatments on day 7 (P = 0.04), no significant pH effect was observed on day 28. The *tet*(W) abundance under initial pH 12 shock was less than other pH shocks on day 28 (P = 0.01), while no temperature effect was observed on day 28. There was no significant temperature and initial pH effect on *cfx*A abundance on each time point during incubation, implying that the bacteria that carrying *cfx*A gene might not be sensitive to these environmental factors. Overall, directly raising temperature and pH can facilitate

pirlimycin removal and decrease *mef*A and *tet*(W) relative abundances during storage of manure slurries.

Key words: antibiotic resistance genes; dairy cow; manure slurry; pirlimycin; pH; temperature

1. Introduction

Antibiotics are widely used for therapeutic and prophylactic purposes in animal husbandry. In 2017, approximately 10.93 million kg of antimicrobial drugs were sold in the United States for use in food-producing animals (USFDA, 2018). Previous studies indicated that up to 90% of administrated antibiotics are eliminated from animal body as parent compounds or metabolites through feces or urine (Kemper, 2008; Ray et al., 2014a). There is strong evidence that antibiotic use in livestock can increase the levels of antibiotic resistant bacteria (ARB) and resistant genes in manure (Koike et al., 2017; Tang et al., 2017). Extensive or inappropriate use of antibiotics can therefore lead to the release of unmetabolized antibiotics and their metabolites as well as antibiotic resistant microorganisms and genes into the environment via manure land application or accidental releases (Aga et al., 2016; Chen et al., 2018; Noyes et al., 2016; Wind et al., 2018). Once in the environment, ARB can proliferate and potentially transfer ARGs to human commensal or pathogenic bacteria via horizontal gene transfer mediated by mobile genetic elements (Berendonk et al., 2015; Guo et al., 2017; Partridge et al., 2018), thus contributing to the global spread of antibiotic resistance.

Around 50% of veterinary antibiotics sold in the United States were used in cattle (USFDA, 2018). Pirlimycin is a class of lincosamide antibiotic widely used in dairy cows for the treatment of clinical and subclinical mastitis infections caused by microorganisms such as *Staphylococcus aureus* and *Streptococcus agalactiae* (Gillespie et al., 2002). Pirlimycin has a bacteriostatic function, binding to the 23S RNA of the 50S ribosomal subunit to inhibit the

peptidyl transferase reaction (Tenson et al., 2003). Macrolide, lincosamide, and streptogramin (MLS) antibiotics have a similar bacteriostatic mode of action, despite distinct chemical structures (Martel et al., 2003). Similar mechanisms of resistance have evolved in bacteria to counter MLS antibiotics (Roberts et al., 1999). The most frequently found macrolide ARGs are the erythromycin ribosome methylation (*erm*) and macrolide efflux (*mef*) genes, which have been widely detected in *Streptococcus* strains (Arpin et al., 1999; Clancy et al., 1996; Daly et al., 2004). Clancy et al. (1996) indicated that the host bacterial E. coli containing the *mef* class A (*mef*A) determinant maintains lower levels of intracellular erythromycin than isogenic clones without *mef*A, primarily due to an active efflux of antibiotics across the cell surface (Paulsen et al., 1996; Sutcliffe et al., 1996). Recently, *mef*A has been detected in dairy manure (Hurst et al., 2019; Muurinen et al., 2017; Noyes et al., 2016) along with predominant manure ARGs such as tetracycline, polypeptide, and β-lactam ARGs (Hurst et al., 2019; Miller et al., 2016; Muurinen et al., 2017; Sun et al., 2016) or in manure-amended soils (Fahrenfeld et al., 2014; Wind et al., 2018).

Manure management practices such as composting and anaerobic digestion have been reported in previous studies as means to reduce antibiotic residues and dissemination of ARGs before their entry to the soil environment (Qian et al., 2016; Sun et al., 2016). Sun et al. (2016) showed that thermophilic anaerobic digestion (55 ºC) could remove 8/10 detected ARGs. Qian et al. (2016) indicated that thermophilic composting process had important role in reducing ARGs and integrons. Gou et al. (2018) demonstrated that aerobic composting significantly reduced the diversity and abundance of ARGs and mobile genetic elements in cattle manure. However, complicated technical requirements limit implementation of manure composting and anaerobic digester operations in many farms (Garfí et al., 2016; Pandyaswargo and

79 Premakumara, 2014). There is an urgent need to develop efficient and sustainable manure
80 handling methods that are appropriate for different farming systems under varying socio-
81 economic environments.

82 Physicochemical factors, such as temperature and pH, likely affect changes of antibiotic
83 concentrations and ARGs abundance during manure composting and anaerobic digestion
84 treatments. Loftin et al. (2008) reported that the chemical stability of antibiotics (such as
85 chlortetracycline, oxytetracycline, tetracycline, and tylosin A) could be affected by temperature
86 (7, 22, and 35°C) and pH (2, 5, 7, 9, and 11). Nicholson et al. (2005) indicated that E. coli,
87 Salmonella, and Campylobacter could survive in stored slurries for up to three months, while all
88 these pathogens could not be detected after approximately one week when temperatures were
89 greater than 55 ºC. Heinonen-Tanski et al. (2004) reported that a dose of 10 g/L of hydrated lime
90 with good stirring could destroy coliform bacteria in diluted cattle slurries to a level below the
91 detection limit. Jamal et al. (2011) found that adding lime to sewage sludge could raise the pH to
92 12 or higher and inactive a high amount of fecal coliforms within one hour. Sun et al. (2016)
93 reported that bacterial phylum Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes are
94 negatively correlated with temperature, while Chloroflexi and Thermotogae are positively
95 correlated with temperature. Rousk et al. (2010) showed that growth of phylum Bacteroidetes
96 and Proteobacteria is positively correlated with pH while growth of Actinobacteria is negatively
97 correlated with pH. Considering that both temperature and pH are critical factors to affect
98 microbial growth (Zhu, 2000), changing temperature or pH during storage of manure slurries
99 might be able to remove antibiotics and to mitigate antibiotic resistance through shifting the
100 microbial community composition away from microbes carrying ARGs. However, no systematic
101 evaluation of the effect of temperature or pH during storage of manure slurries has been

conducted. The objective of this study was to determine the fate of pirlimycin and ARGs in dairy manure slurries in response to initial pH (5, 7, 9, and 12) shocks and temperatures (10, 35, and 55ºC) at which the manure is stored. We hypothesized that a simple change of temperature or initial pH would affect the removal of pirlimycin and prevalence of ARGs in dairy manure slurries.

2. Materials and methods

2.1.Manure generation

To generate dairy manure for incubation studies, five healthy lactating dairy cows with similar age, body weight, and stage in lactation cycle were selected and housed individually in metabolic stalls at the Virginia Tech Dairy Center (Blacksburg, VA). The animal trial was conducted in accordance with the Federation of Animal Science Societies' Guide for the Care and Use of Agricultural Animal in Research and Teaching, and approved by the Virginia Tech Institutional Animal Care and Use Committee. All cows had free access to water and were fed a total mixed ration formulated according to National Research Council nutrient recommendations (NRC, 2001) to meet all nutrient requirements. Formulated ingredients and nutrient compositions of the total mixed ration are listed in Table A1 (Supplement). Herd medication records indicated that none of the cows had received antibiotic treatment during the current lactation period (at least 200 days). Pirlimycin was administered via intramammary infusion at the recommended dose for clinical mastitis - two doses of 50 mg each administered 24 h apart (Pharmacia & Upjohn Company, Division of Pfizer Inc., New York). Prior to the pirlimycin administration, cows were fitted with urinary catheters for total urine collection. Lidocaine jelly (2-3 ml; Akorn, Inc., Lake Forest, IL) was applied intravaginally and to the catheters to minimize discomfort. After the first dose of treatment, total collection of feces and urine was conducted on day 1 to

day 4 and excreta from all cows was mixed completely to yield a large homogenous pool of manure for the incubation experiments. The concentration of pirlimycin in the mixture was 172.5 ng/g (dry weight based). Rectal temperature was monitored, and no symptoms of urinary infections were observed during the experimental period.

2.2.Manure slurry incubation

Manure slurry was generated by mixing manure and sterile water to achieve a final solid content of 5% to mimic the solid content in a typical manure slurry storage lagoon. Aliquots (200 mL) of slurry were transferred into 400 mL glass beakers. The initial manure slurry pH was 7.5. To simulate psychrophilic, mesophilic, and thermophilic environments, beakers were incubated at 10, 35, or 55ºC without initial pH adjustment for 90 days. Similarly, slurry samples in beakers were incubated at consistent ambient temperature 25ºC for 90 days with initial pH adjusted to 5, 7, 9, or 12 using 1 M HCl or NaOH. Each treatment had 4 replicates. Beakers were covered with aluminum foil with a hole in the middle to maintain aerobic conditions, weighed daily, and weight loss due to water evaporation was replaced with sterilized water. Manure slurry was totally mixed using a clean glass rod, and 2 mL was sampled from each beaker on day 0, 1, 3, 7, 14, 28, 56, and 90 to monitor pirlimycin concentrations. Samples were stored at -20ºC until analysis for pirlimycin. On day 0, 3, 7, and 28 additional 2 mL sample was collected, immediately freeze dried, and stored at -80ºC for future DNA extraction.

2.3.Quantification of pirlimycin

Pirlimycin in manure slurries was extracted, cleaned up, and analyzed using the method described by Ray et al. (2014b). Briefly, ~1 g wet manure sample was freeze-dried and then extracted using methanol-phosphate buffer and cleaned up using a solid-phase extraction (SPE) set up, which includes an OASIS HLB (hydrophilic-lipophilicbalanced) with a short cartridge

(250 mg sorbent, Waters Corp., Milford, MA) fitted in a 20 port SPE vacuum manifold (Agilent Technologies, Lexington, MA). The cleaned-up extract was analyzed for pirlimycin using Agilent 1290 ultra-performance liquid chromatography (UPLC) coupled with Agilent 6490 Triple Quad tandem mass spectrometer (Agilent Technologies, Santa Clara, CA).

2.4.DNA extraction and quantitative Real-Time Polymerase Chain Reaction (q-PCR)

About 100 mg freeze-dried manure were weighed and DNA was extracted using the QIAamp DNA stool extraction kit (Qiagen, Valencia, CA), according to the manufacturer's instructions.

Real-time PCR was performed to quantify a macrolide ARG (*mef*A), a tetracycline ARG (*tet*(W)), and a β-lactam ARG (*cfx*A) using an EvaGreen assay with previously published primers (Aminov et al., 2001; Looft et al., 2012; Sóki et al., 2011). These three ARGs were chosen because our prior metagenomic analysis study demonstrated that tetracycline, MLS, and β-lactam antibiotic resistance genes were the most dominant ARGs in feces from the dairy cows treated with pirlimycin, and the pirlimycin administration increased the abundance of these genes compared with the feces from untreated cows (Caudle, 2014), so an effect of temperature or pH on the three ARGs was expected. To normalize the quantities of each ARG to the total bacterial population, the bacterial 16S rRNA gene was quantified using the approach as described by Suzuki et al. (2000). Standard solutions were made from a 10-fold serial dilution of a cloned gene ranging from 10^8 to 10^2 gene copies/µl. Based on the standard solutions, standard curves were constructed to quantify gene copies of each ARG. Samples were analyzed in triplicate with a standard curve and a negative control in each run.

2.5.Statistical analysis

Data processing and analyses were conducted in R software (R development Core Team, 2015). Pirlimycin concentration was calculated based on dry manure weight (unit is ng/g). ARGs were normalized to gene copies per bacterial 16S rRNA gene and then natural log transformed. One-way analysis of variance (ANOVA) was carried out to test manure slurry incubation temperature or initial pH effect on pirlimycin concentrations and ARG abundances at each time point using the lm function in R. Multiple paired comparisons were conducted using the Tukey's honestly significant difference (HSD). Significant differences were declared at $P < 0.05$.

3. Results and discussion

3.1. Temperature effect on the removal of pirlimycin

Pirlimycin concentrations in response to incubation time at different temperatures without adjusting initial pH are displayed in Figure 1. The average pirlimycin concentration in the manure slurries was 175.8 ng/g (dry matter based) on day 0. Within 1 day of incubation, the levels of pirlimycin at the thermophilic environment immediately increased to 1378.1 ng/g, which was 8 times greater than the initial value in the manure slurry. However, within this time period at the mesophilic and psychrophilic conditions, the levels of pirlimycin were close to its initial concentration. Therefore, pirlimycin concentration at the thermophilic environment was greater than that at the mesophilic and psychrophilic conditions on day 1 ($P < 0.001$). Beyond the first day incubation at the thermophilic condition, pirlimycin concentration rapidly decreased from day 1 to day 28 and then remained at similar levels until the end of 90-day incubation, at which time its concentration was 0.2 times less than that of day 0. In contrast to the thermophilic condition, the pirlimycin concentration at the psychrophilic condition slowly increased over several weeks, reaching its peak on day 56 at 4.3 times greater than the initial concentration. The pirlimycin concentration slowly decreased thereafter, but on day 90 it remained 3.1 times of the

concentration on day 0. Although the concentration pattern of pirlimycin at the mesophilic environment was similar to that incubated at the psychrophilic condition, compared to the psychrophilic condition, the mesophilic condition had a greater increase rate within the first 14 days (peaking at 7.7 times of the initial concentration on day 14) and a greater decrease rate thereafter until day 90. Due to the different concentration patterns of pirlimycin over time, pirlimycin concentration at the mesophilic and thermophilic conditions was greater than its levels at the psychrophilic condition on day 3 and 7 ($P < 0.05$), and pirlimycin concentrations incubated at the thermophilic condition were less than that at the mesophilic and psychrophilic conditions on day 28, 56, and 90 ($P = 0.04$, < 0.001, and 0.01).

The observed increase in pirlimycin concentration in the early stages (1 to14 days) of incubation was likely due to deconjugation of pirlimycin conjugates back to pirlimycin. Hornish et al. (1998) indicated that pirlimycin was converted to pirlimycin sulfoxide and pirlimycin sulfone conjugates in bovine liver. A similar deconjugation phenomenon in raw manure-amended soils was reported by Chen et al. (2018). Hornish et al. (1992) demonstrated that approximately 50% of pirlimycin was excreted through milk, and 34% of pirlimycin was released via feces and urine following pirlimycin intramammary administration. In the current study, in total 100 mg pirlimycin was injected into teat canal, and on average 24 kg manure (dry matter based) was totally collected from day 1 to day 4 for each cow. The maximum pirlimycin concentration is 1417 ng/g in the manure mixture assuming 34% elimination rate in manure and no formation of conjugates. The estimated maximum concentration was consistent with the peak pirlimycin concentration at the thermophilic environment on day 1. Based on the initial pirlimycin concentration and the estimated maximum concentration, one could speculate that pirlimycin conjugates accounted for roughly 87% of pirlimycin residues (including pirlimycin

and its conjugated forms) in manure. Apparently, the deconjugation of pirlimycin conjugates was predominant in the early stages, and the thermophilic environment facilitated the deconjugation, while the psychrophilic and thermophilic conditions delayed the deconjugation compared to the thermophilic condition. Along with deconjugation of pirlimycin conjugates, pirlimycin and its conjugates can be simultaneously degraded through both abiotic and biotic processes (Aga et al., 2016; Hornish et al., 1992; Ray et al., 2017). Loftin et al. (2008) indicated that lincomycin type antibiotics were rarely hydrolyzed at temperature relevant to environmental conditions because of its recalcitrant linkage structure. However, Hornish et al. (1992) demonstrated that microbes in the digestive tract of dairy cows converted pirlimycin and pirlimycin sulfoxide conjugate to ribonucleotide adducts. Thus, the removal of pirlimycin and its conjugates by fecal microbes likely explains the eventual decreased pirlimycin concentrations across temperature treatments over time.

Ray et al. (2017) showed that the transformation of pirlimycin during composting relies largely on temperature. In the current study, after the initial concentration increase due to deconjugation, thermophilic conditions increased the removal of pirlimycin compared to the psychrophilic and mesophilic conditions. This resulted in a significant decrease of pirlimycin concentrations on day 28, 56, and 90 at the thermophilic condition. Overall, although the thermophilic condition initially resulted in a sharp increase of pirlimycin in the manure slurries compared to the other two lower temperature conditions, it is effective for the overall pirlimycin removal if manure is treated at this temperature for up to 28 days. Storage or treatment of manure from pirlimycin treated animals at psychrophilic and mesophilic conditions are not recommended, regardless of the length of manure storage, if the goal is to remove pirlimycin from the manure.

3.2. Initial pH effect on the removal of pirlimycin

Pirlimycin concentrations in response to incubation time at different initial pH with consistent ambient temperature 25 ºC are shown in Figure 2. On average, the initial pirlimycin concentration was 171.5 ng/g. As occurred during incubation of manure slurry samples at varying temperature, pirlimycin concentrations increased to a plateau on day 7 at initial pH 9, and on day 28 at initial pH 7 and 12 (at concentrations 5.8, 4.7, and 5.0 times greater than initial, respectively), suggesting that the deconjugation of pirlimycin conjugates was more dominant than removal in the early stages (1 to 28 days). After that, degradation began to surpass deconjugation of pirlimycin conjugates, resulting in a decrease of pirlimycin concentrations until the end of 90-day incubation. Because of the changes of deconjugation and removal over time among pH treatments, pirlimycin concentrations at pH 7 and 9 shocks were greater than that at pH 12 on day 3 and 7 ($P < 0.05$). However, no significant difference of pirlimycin concentration was observed among the neutral and alkaline shocks on day 14, 28, and 56. Although pirlimycin concentrations at the alkaline shocks were less than the neutral shock on day 90 ($P < 0.05$), their concentrations were still greater than initial concentrations by 2.1-fold and 1.8-fold, respectively, at pH 9 and 12 shocks. At the acidic shock, the pirlimycin concentrations significantly increased within the first 3 days, resulting in a greater pirlimycin concentration than the other pH treatments on day 3 ($P < 0.05$). Instead of a decrease after reaching a plateau, the pirlimycin concentrations (around 1100 ng/g) remained 6.8 times of the initial level (161.8 ng/g) on day 56 and day 90, which was greater than the rest of pH treatments ($P < 0.05$). The peak pirlimycin concentration under the acidic shock was close to the estimated maximum pirlimycin concentration (1417 ng/n). This suggests that the acidic shock facilitated release of pirlimycin from conjugated compounds but completely hindered removal of pirlimycin

262 in dairy manure slurries. This result was consistent with a previous study, as Baroody et al.
263 (2000) reported that clindamycin (also a lincosamide) had a maximum stability at pH 4 to 5 with
264 20% removal rate after one month in pH ranging from 4 to 7 at 40ºC.
265 To the best of our knowledge, no previous study has reported the stability of pirlimycin in
266 manure exposed to different pH conditions; our results indicate pirlimycin residues are
267 susceptible to removal in alkaline conditions in dairy manure. A similar compound lincomycin
268 was not degraded at pH 5, 7, and 9 in surface water, anaerobic swine lagoons, wastewater, and
269 ground water (Loftin et al., 2008). Oesterling (1970) observed that lincomycin was stable over
270 time at pH values greater than 5. Although pirlimycin and lincomycin all consist of three
271 components (an amino acid, a sugar, and an amide bond), pirlimycin is a semi-synthetic
272 derivative of lincomycin. The difference of their conformational landscape and electron densities
273 might determine the physicochemical properties in response to varied pH. In the current study,
274 pirlimycin appears easier to remove in alkaline conditions than the acidic condition, which is
275 supported by a previous study, as the chemical structure of pirlimycin is stable at low pH
276 condition (Crow et al., 1999).
277 Chen et al. (2018) reported that the half-life for pirlimycin in raw manure-amended soils
278 ranged from 5.5 to 8.2 days. Wind et al. (2018) showed that pirlimycin was quickly dissipated
279 within 29 days in dairy manure-amended soils. In the current study, the removal of pirlimycin
280 did not fit either single phase or bi-phase first order kinetics. However, across different
281 temperature and pH conditions (except for the thermophilic environment), the concentration of
282 pirlimycin were greater than 300 μg/g on day 90 implying that the removal of pirlimycin was
283 slower in the manure slurries than in the manure-amended soils. The difference could be caused
284 by the different microbial community and activity (Cleary et al., 2016; Lertpaitoonpan, 2008),

285 and/or sorption. Pirlimycin can be absorbed to either clay content or organic matter in soils
286 (Ötker and Akmehmet-Balcıoğlu, 2005; Sandegren, 2014; Wind et al., 2018), leading to quickly
287 decreased pirlimycin concentrations in manure-amended soils.

288 ***3.3. Bacterial growth in response to various temperature and pH***

289 The mesophilic and thermophilic environment increased total 16S gene copies per g dry
290 manure compared to the psychrophilic environment on day 3, 7, and 28 (P = 0.04, < 0.001, and
291 0.05; Figure 3 and Table 1), indicating that, as expected, higher temperature exerted a positive
292 influence on bacterial growth in manure slurries. While varying across bacterial species, the
293 optimum growth temperature is around 37ºC (Zhu, 2000). Nedwell (1999) demonstrated that the
294 affinity of microorganisms for substrates was decreased consistently when temperature dropped
295 below the optimum temperature. Therefore, at the psychrophilic condition, bacteria become
296 increasingly unable to sequester nutrients from the natural environment for maintenance and
297 growth, resulting in decreased bacterial population.

298 In the current study, there was no significant difference in bacterial 16S gene copies on
299 day 3 and 28 at the mesophilic and thermophilic conditions (Table 1 and Figure 3), although
300 temperature might have changed the dynamics of bacterial growth and death. Previous studies
301 indicated that thermophilic conditions could offer more advantages such as higher metabolic
302 rates, and higher growth rates compared to bacteria in mesophilic conditions but also higher
303 death rates (Duran and Speece, 1997; van Lier, 1995).

304 Lin et al. (2013) investigated initial pH (3-12) effects on hydrolysis and acidification
305 reactions in manure and found that initial pH shifted the microbial community structure
306 composition and consequent fermentation products. In the current study, no significant initial pH
307 effect was observed for bacterial 16S gene copies per g dry manure over the whole incubation

period, implying that total bacterial amount was not affected by pH although the microbial community might have been changed. Bacterial 16S gene copies tended to increase over time, suggesting that the bacterial population increased during storage of manure slurries regardless of varied initial pH shocks, though a previous study indicated the optimum pH for bacterial growth is neutral or near neutral pH (Zhu, 2000).

3.4. Temperature and initial pH effects on the prevalence of *mef*A

The *mef*A relative abundance linearly declined over time within each of the temperature treatments (Table 1 and Figure 3). No significant temperature effect on *mef*A abundance was observed on day 3. However, compared to the psychrophilic and mesophilic environments, the thermophilic reactors had decreased *mef*A relative abundance on day 7 and 28 (P = 0.02 and 0.04). There was no *mef*A abundance difference between the psychrophilic and mesophilic environment on day 7, but on day 28 *mef*A relative abundance was greater at the mesophilic environment than the psychrophilic environment.

Previous studies showed that the variations of ARGs were associated with changes in the bacterial community (Qian et al., 2016; Sun et al., 2016). Remarkable differences in the bacteria community between high temperature (55ºC) and moderate temperature (20-35ºC) have been reported in a previous study (Cho et al., 2015). Miller et al. (2016) demonstrated that high temperature could eliminate some ARG hosts that were not thermotolerant. Hurst et al. (2019) reported that the *mef*A gene abundance was 4-fold higher in spring storage dairy manure samples than fall storage samples, implying a temperature effect. Sun et al. (2016) found that thermophilic anaerobic digestion performed better at reducing ARGs than moderate and mesophilic digestion. In the current study, *mef*A decreased 4.2-fold on day 28 relative to day 0 under the thermophilic environment, while it decreased 2.87-fold and 1.14-fold under

psychrophilic and mesophilic conditions, suggesting that high temperature facilitated the removal of host bacteria that encoded the *mef*A gene.

Different prevalence patterns of *mef*A relative abundance in response to different pH shocks during manure slurry incubation were observed (Table 2 and Figure 4). Under acidic shock at pH 5, the relative abundance of *mef*A decreased over time, while it increased at pH 9 over time until day 7 and decreased thereafter. Similar prevalence pattern of *mef*A relative abundance over time was observed at pH 7 and 12 shocks. It rapidly increased on day 3 and decreased thereafter until day 28. The *mef*A relative abundance was greater at the pH 9 shock than the rest of pH treatments on day 7 ($P = 0.04$). However, no significant pH treatment effect was observed on *mef*A relative abundance on day 3 and 28.

Lin et al. (2013) reported that initial pH shifted the microbial community in swine manure. Although there was no significant pH effect on the relative abundance of *mef*A in dairy manure except for day 7, we did find a difference in prevalence patterns of *mef*A among pH treatments. The *mef*A relative abundance was continuously decreased under the acidic treatment, but it was increased under the neutral and alkaline shocks in the early stages, implying that the acidic condition inhibited the growth of *mef*A host bacteria compared to the environment at pH 9. However, limited information is available regarding pH effect on dissemination of *mef*A. More research is needed to investigate bacteria related to the *mef*A gene enrichment and their fate in response to pH treatments.

3.5. Temperature and initial pH effects on the prevalence of tet(W)

The relative abundance of *tet*(W) rapidly increased from day 0 to day 3 regardless of the temperature treatment, and then decreased thereafter on day 3 at the psychrophilic and mesophilic environment, but remained relative high until day 7 and decreased thereafter at the

thermophilic environment (Figure 3). On day 3, both the psychrophilic and thermophilic environment exhibited a greater *tet*(W) relative abundance than the mesophilic environment ($P < 0.05$; Table 1). However, the *tet*(W) relative abundance at the thermophilic environment was greater than the psychrophilic and mesophilic environment on day 7, and no significant difference was observed among three treatments on day 28.

The *tet*(W) gene encodes for ribosome protection proteins residing on mobile or conjugative elements (Roberts, 1996), which has been detected in both gram-positive and gram-negative bacteria, especially from samples isolated from wastewater sources (Miller et al., 2016; Storteboom et al., 2010). Although, Sun et al. (2016) reported that there was no temperature effect on *tet*(W) in dairy manure during anaerobic digestion, it was supervising that higher temperature increased *tet*(W) relative abundance compared to the mesophilic environment, as Miller et al. (2016) showed that the *tet*(W) relative abundance in wastewater sludge remained consistently low in the thermophilic digester, which was less than in the mesophilic digester. Qian et al. (2016) indicated that high temperature could enhance the degradation of organics, such as antibiotics and hormones, to impose a selective or co-selective pressure on ARGs. In the present study, the thermophilic environment significantly decreased pirlimycin concentrations, resulting in an increase of *tet*(W) relative abundance by imposing less selective pressure on resistant bacteria that encoded *tet*(W).

The relative abundance of *tet*(W) at pH 7 shock was significantly increased on day 3 and decreased thereafter until day 28, while it increased at pH 5, 9, and 12 shocks until day 7 and then decreased thereafter (Table 2 and Figure 4). The *tet*(W) relative abundance was greater at pH 7 shock than the rest of the pH treatments on day 3 ($P = 0.03$). However, no significant difference was observed among pH treatments on day 7. On day 28, the relative abundance of

377 *tet*(W) at pH 12 shock declined 3.67 order of magnitude relative to day 0, which was less than
378 the rest of the pH treatments (P = 0.01).

379 As discussed in the previous section, pirlimycin might impose selective pressure on
380 tetracycline resistant bacteria at different temperature conditions. However, this speculation was
381 not consistent with the results from different pH treatments. We observed high pirlimycin
382 concentrations at pH 5 shock during incubation time, but the *tet*(W) relative abundance was not
383 decreased consequently. This might reflect an interaction between pH and pirlimycin on bacterial
384 community and growth. Greater *tet*(W) abundance was observed at pH 9 shock than other
385 treatments on day 3, indicating that pH 9 was the optimum pH for *tet*(W) resistant bacteria
386 growth in the early stages. However, the significant decrease at pH 12 shock on day 28 implies
387 that the *tet*(W) host bacteria were susceptible to the alkaline shock at pH 12.

388 ***3.6.Temperature and initial pH effect on the prevalence of*** *cfx*A

389 There was no significant temperature or pH effect on the relative abundance of *cfx*A on
390 each time point during incubation of manure slurries. The relative abundance of *cfx*A decreased
391 with time irrespective to the varied temperature or pH treatment (Figure 3 and Figure 4).

392 The *cfx*A gene encodes class A β-lactamase which has high capacity to hydrolyze
393 cephaloridine and cephalothin (García et al., 2008). Zhou et al. (2016) reported that *cfx*A is the
394 most abundant ARG observed in dairy manure. However, in the current study, the relative
395 abundance of *cfx*A was less than *mef*A and *tet*(W), which might reflect different antibiotic uses
396 and contact histories among dairy farms. There was no temperature or initial pH effect on *cfx*A
397 abundance, indicating that the bacteria that carrying *cfx*A gene might not be sensitive to these
398 environmental factors or potential pirlimycin selective pressure. However, a decrease of *cfx*A

relative abundance was observed over time in both temperature and pH groups, implying that prolong the storage time could decrease *cfx*A gene abundance in manure slurries.

4. Conclusion

We investigated the effects of temperature (10, 35, and 55ºC) and initial pH (5, 7, 9, and 12) on the dissipation of pirlimycin and prevalence of ARGs in dairy manure slurries. Our results indicated that less complicated manure management practices, such as raising temperature of manure slurry to 55 ºC without changing pH or increasing its pH to 12 at ambient temperature are recommended during storage of manure slurries to facilitate the removal of pirlimycin residues and the reductions of *mef*A and *tet*(W) gene abundances. In practice, if it is too expensive or too difficult to increase temperature during storage of manure slurries in different farm systems, simply adjusting pH at ambient temperature can be an optimal strategy to reduce risk with land application of antibiotic-laden manure. Our findings have important implications for understanding the impacts of physicochemical factors on removal of antibiotics in manure slurries, and necessitate future studies to examine their effects of on a wide spectrum of ARGs in diverse types of animal manures.

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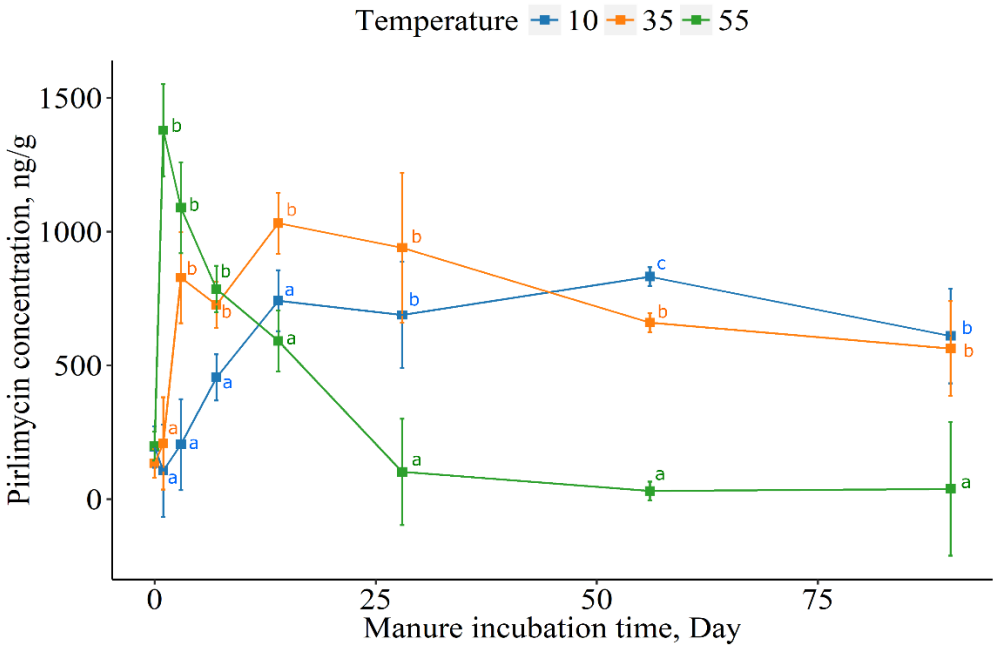

Figure 1. Pirlimycin concentrations in dairy manure slurries in response to incubation time at different temperatures with same initial pH 7.5. The pirlimycin concentration in the slurry on day 0 was 175.8 ng/g. Error bars represent mean of standard error of four replicates (n = 4). Different characters within the same incubation time indicate significantly different means.

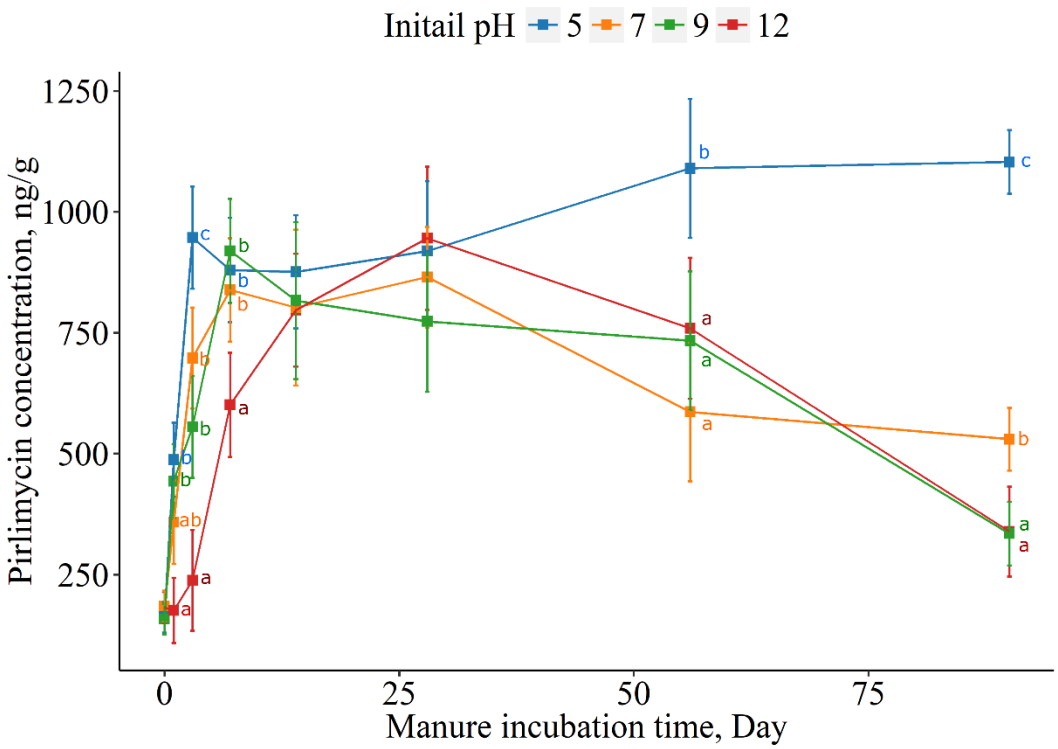

Figure 2. Pirlimycin concentrations in dairy manure slurries in response to incubation time at different pH shocks with consistent ambient temperature 25 ºC. The pirlimycin concentration in the slurry on day 0 was 171.5 ng/g. Error bars represent mean of standard error of four replicates (n = 4). Different characters within the same incubation time indicate significantly different means.

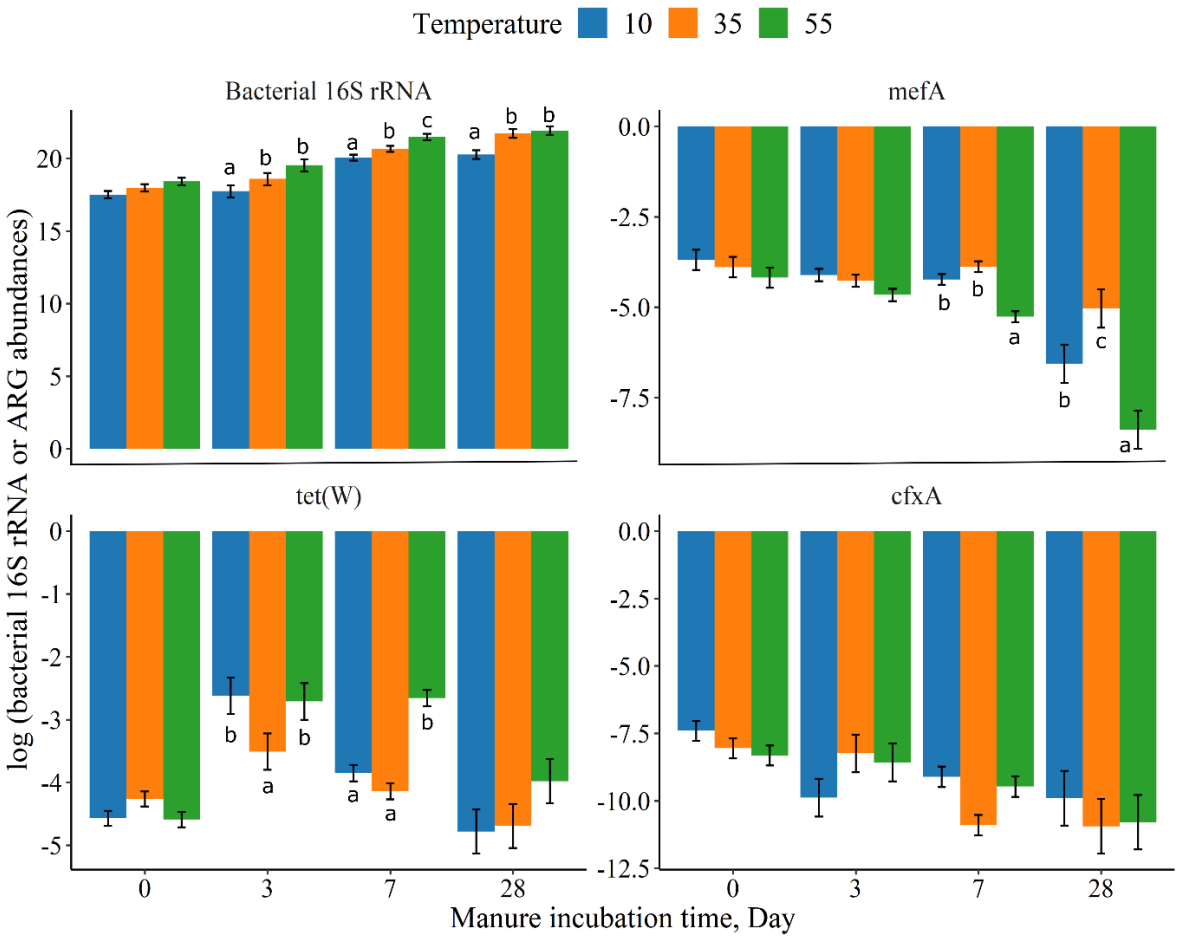

Figure 3. The bacterial 16S rRNA, and ARG abundances in dairy manure slurries in response to incubation time at different temperatures with same initial pH 7.5. Bacterial 16S rRNA expressed as total copy numbers per g dry manure. ARGs are reported in terms of relative abundance, normalized to bacterial 16S rRNA copy numbers. Natural log transformation was used to normalize the bacterial 16S rRNA, and ARG abundances. Error bars represent mean of standard error of four replicates (n = 4). Different characters within the same incubation time indicate significantly different means.

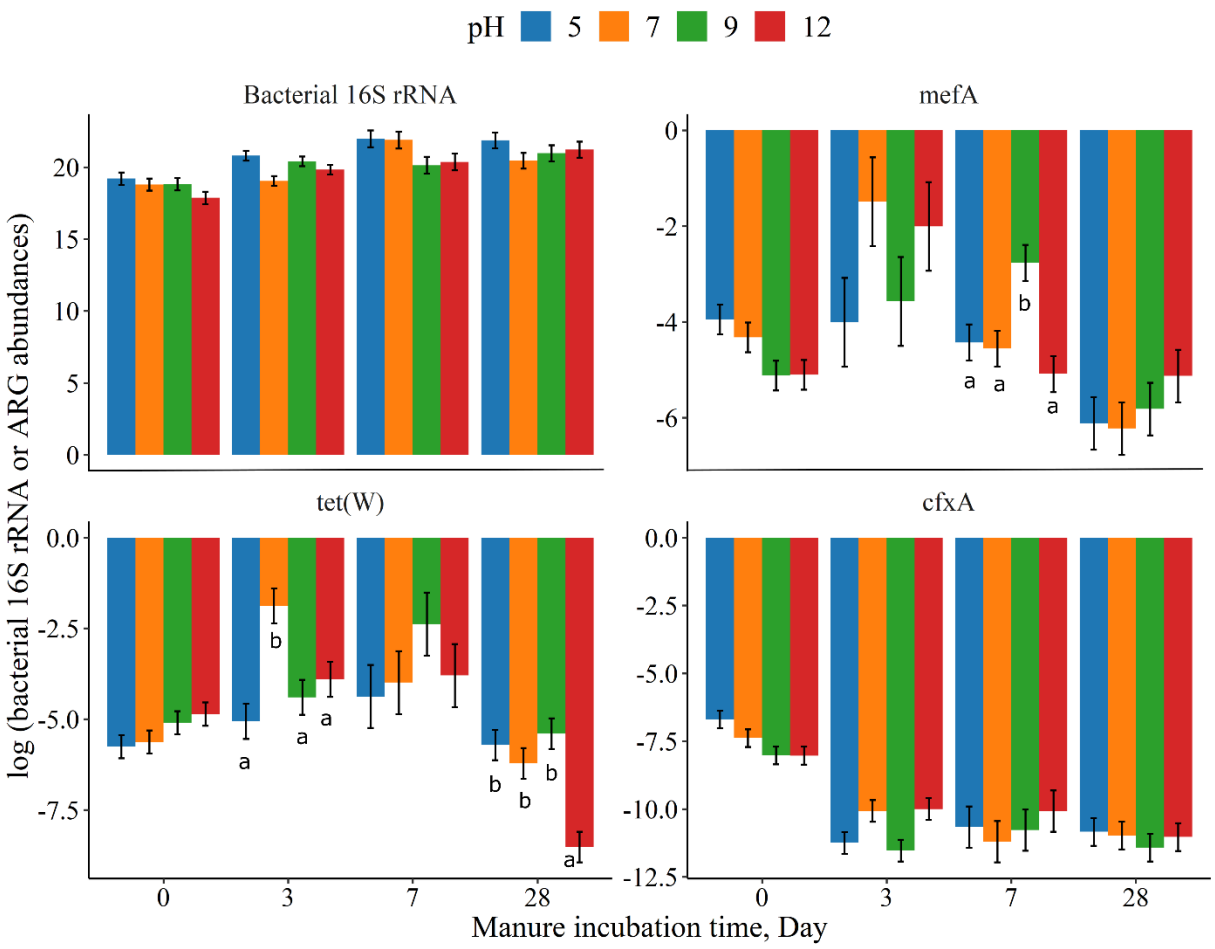

Figure 4. The bacterial 16S rRNA, and ARG abundances in dairy manure slurries in response to incubation time at different pH shocks with consistent ambient temperature 25 ºC. Bacterial 16S rRNA expressed as total copy numbers per g dry manure. ARGs are reported in terms of relative abundance, normalized to bacterial 16S rRNA copy numbers. Natural log transformation was used to normalize bacterial 16S rRNA, and ARG abundances. Error bars represent mean of standard error of four replicates (n = 4). Different characters within the same incubation time indicate significantly different means.

613 Table 1. Effect of temperature on pirlimycin concentrations, bacterial 16S rRNA, and ARG
614 abundances in dairy manure slurries[1]

Temperature	10ºC	35ºC	55ºC	SEM[2]	*P* value
Pirlimycin, ng/g					
Day 0	194.75	134.18	198.33	29.71	0.24
Day 1	106.44^{a}	208.61^{a}	1378.10^{b}	83.00	< 0.001
Day 3	204.24^{a}	827.85^{b}	1088.71^{b}	85.32	< 0.001
Day 7	454.92^{a}	726.18^{b}	784.85^{b}	44.48	0.001
Day 14	741.67^{a}	1032.80^{b}	590.80^{a}	58.22	0.001
Day 28	686.68^{b}	924.75^{b}	95.56^{a}	102.83	0.04
Day 56	831.64^{c}	660.39^{b}	30.48^{a}	17.58	< 0.001
Day 90	609.88^{b}	563.36^{b}	38.09^{a}	83.77	0.01
Bacterial 16S rRNA					
Day 0	17.51	17.98	18.42	0.25	0.09
Day 3	17.74^{a}	18.59^{b}	19.53^{b}	0.42	0.04
Day 7	20.07^{a}	20.67^{b}	21.49^{c}	0.21	< 0.001
Day 28	20.28^{a}	21.73^{b}	21.92^{b}	0.30	0.05
*mef*A					
Day 0	-3.69	-3.89	-4.18	0.28	0.53
Day 3	-4.11	-4.27	-4.66	0.17	0.20
Day 7	-4.23^{b}	-3.88^{b}	-5.26^{a}	0.15	0.02
Day 28	-6.56^{b}	-5.03^{c}	-8.38^{a}	0.53	0.04
tet(W)					
Day 0	-4.57	-4.26	-4.59	0.12	0.06
Day 3	-2.62^{b}	-3.51^{a}	-2.71^{b}	0.29	0.04
Day 7	-3.85^{a}	-4.14^{a}	-2.66^{b}	0.13	0.01
Day 28	-4.78	-4.69	-3.98	0.35	0.34
*cfx*A					
Day 0	-7.40	-8.05	-8.32	0.37	0.33
Day 3	-9.88	-8.24	-8.57	0.70	0.35
Day 7	-9.11	-10.90	-9.47	0.38	0.09
Day 28	-9.90	-10.94	-10.79	1.01	0.75

615 [1]Bacterial 16S rRNA expressed as total copy numbers per g dry manure; ARGs are reported in
616 terms of relative abundance, normalized to bacterial 16S rRNA copy numbers. Natural log
617 transformation was used to normalize bacterial 16S rRNA, and ARG abundances. Different
618 superscripts in the same row indicate significantly different means.

619 [2]Standard error of mean

620 Table 2. Effect of initial pH on pirlimycin concentrations, bacterial 16S rRNA, and ARG
621 abundances during dairy manure slurries[1]

Initial pH	5	7	9	12	SEM[3]	*P* value
Pirlimycin, ng/g						
Day 0	161.82	181.15	157.85	185.27	16.55	0.58
Day 1	487.46^{b}	354.68^{ab}	443.52^{b}	178.19^{a}	41.01	< 0.001
Day 3	946.21^{c}	683.53^{b}	553.70^{b}	249.32^{a}	55.88	< 0.001
Day 7	880.83^{b}	852.27^{b}	925.54^{b}	596.64^{a}	55.30	0.005
Day 14	871.10	792.29	806.81	801.57	72.59	0.82
Day 28	924.11	860.00	757.57	925.19	66.39	0.33
Day 56	1101.26^{b}	587.43^{a}	751.98^{a}	777.95^{a}	55.22	0.01
Day 90	1102.38^{c}	530.25^{b}	333.55^{a}	339.90^{a}	26.10	< 0.001
Bacterial 16S rRNA						
Day 0	19.21	18.80	18.82	17.87	0.39	0.24
Day 3	20.81	19.05	20.41	19.83	0.34	0.23
Day 7	21.98	21.89	20.14	20.37	0.63	0.06
Day 28	21.87	20.45	20.98	21.23	0.55	0.45
*mef*A						
Day 0	-3.95	-4.33	-5.12	-5.11	0.31	0.06
Day 3	-4.01	-1.49	-3.57	-2.01	0.92	0.30
Day 7	-4.43^{a}	-4.56^{a}	-2.77^{b}	-5.09^{a}	0.38	0.04
Day 28	-6.12	-6.23	-5.82	-5.13	0.55	0.51
tet(W)						
Day 0	-5.76	-5.63	-5.10	-4.86	0.32	0.56
Day 3	-5.06^{a}	-1.88^{b}	-4.40^{a}	-3.90^{a}	0.48	0.03
Day 7	-4.37	-3.99	-2.38	-3.80	0.87	0.48
Day 28	-5.71^{b}	-6.22^{b}	-5.40^{b}	-8.53^{a}	0.42	0.01
*cfx*A						
Day 0	-6.69	-7.38	-8.03	-8.03	0.33	0.12
Day 3	-11.24	-11.07	-11.52	-11.00	0.40	0.11
Day 7	-10.66	-11.20	-10.77	-11.07	0.76	0.95
Day 28	-10.84	-10.97	-11.42	-11.03	0.51	0.92

622 [1]Bacterial 16S rRNA expressed as total copy numbers per g dry manure; ARGs are reported in
623 terms of relative abundance, normalized to bacterial 16S rRNA copy numbers. Natural log
624 transformation was used to normalize bacterial 16S rRNA, and ARG abundances. Different
625 superscripts in the same row indicate significantly different means.

626 [2]Standard error of mean