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1 **Fate of pirlimycin and antibiotic resistance genes in dairy manure slurries in response to**
2 **temperature and pH adjustment**

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10 **Abstract**

11 Quantifying the fate of antibiotics and antibiotic resistance genes (ARGs) in response to
12 physicochemical factors during storage of manure slurries will aid in efforts to reduce the spread
13 of resistance when manure is land-applied. The objectives of this study were to determine the
14 effects of temperature (10, 35, and 55°C) and initial pH (5, 7, 9, and 12) on the removal of
15 pirlimycin and prevalence of ARGs during storage of dairy manure slurries. We collected and
16 homogenized feces and urine from five lactating dairy cows treated with pirlimycin and prepared
17 slurries by mixing manure and sterile water. Aliquots (200 ml) of slurry were transferred and
18 incubated in 400 mL glass beakers under different temperatures (10, 35, and 55°C) or initial pH
19 (5, 7, 9, and 12). Pirlimycin concentration and abundances of 16S rRNA, *mefA*, *tet(W)*, and *cfxA*
20 as indicators of total bacteria and ARGs corresponding to macrolide, tetracycline, and β -lactam
21 resistance, respectively, were analyzed during manure incubation. The thermophilic environment
22 (55°C) increased the deconjugation and removal of pirlimycin, while the acidic shock at pH 5
23 increased deconjugation but inhibited removal of pirlimycin, suggesting that the chemical
24 stability of pirlimycin could be affected by temperature and pH. The thermophilic environment
25 decreased *mefA* relative abundance on day 7 and 28 ($P = 0.02$ and 0.04), which indicates the
26 bacteria that encoded *mefA* gene were not thermotolerant. Although *mefA* relative abundance
27 was greater at the pH 9 shock than the rest of pH treatments on day 7 ($P = 0.04$), no significant
28 pH effect was observed on day 28. The *tet(W)* abundance under initial pH 12 shock was less than
29 other pH shocks on day 28 ($P = 0.01$), while no temperature effect was observed on day 28.
30 There was no significant temperature and initial pH effect on *cfxA* abundance on each time point
31 during incubation, implying that the bacteria that carrying *cfxA* gene might not be sensitive to
32 these environmental factors. Overall, directly raising temperature and pH can facilitate

33 pirlimycin removal and decrease *mefA* and *tet(W)* relative abundances during storage of manure
34 slurries.

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

36 **1. Introduction**

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

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

56 peptidyl transferase reaction (Tenson et al., 2003). Macrolide, lincosamide, and streptogramin
57 (MLS) antibiotics have a similar bacteriostatic mode of action, despite distinct chemical
58 structures (Martel et al., 2003). Similar mechanisms of resistance have evolved in bacteria to
59 counter MLS antibiotics (Roberts et al., 1999). The most frequently found macrolide ARGs are
60 the erythromycin ribosome methylation (*erm*) and macrolide efflux (*mef*) genes, which have
61 been widely detected in *Streptococcus* strains (Arpin et al., 1999; Clancy et al., 1996; Daly et al.,
62 2004). Clancy et al. (1996) indicated that the host bacterial *E. coli* containing the *mef* class A
63 (*mefA*) determinant maintains lower levels of intracellular erythromycin than isogenic clones
64 without *mefA*, primarily due to an active efflux of antibiotics across the cell surface (Paulsen et
65 al., 1996; Sutcliffe et al., 1996). Recently, *mefA* has been detected in dairy manure (Hurst et al.,
66 2019; Muurinen et al., 2017; Noyes et al., 2016) along with predominant manure ARGs such as
67 tetracycline, polypeptide, and β -lactam ARGs (Hurst et al., 2019; Miller et al., 2016; Muurinen et
68 al., 2017; Sun et al., 2016) or in manure-amended soils (Fahrenfeld et al., 2014; Wind et al.,
69 2018).

70 Manure management practices such as composting and anaerobic digestion have been
71 reported in previous studies as means to reduce antibiotic residues and dissemination of ARGs
72 before their entry to the soil environment (Qian et al., 2016; Sun et al., 2016). Sun et al. (2016)
73 showed that thermophilic anaerobic digestion (55 °C) could remove 8/10 detected ARGs. Qian
74 et al. (2016) indicated that thermophilic composting process had important role in reducing
75 ARGs and integrons. Gou et al. (2018) demonstrated that aerobic composting significantly
76 reduced the diversity and abundance of ARGs and mobile genetic elements in cattle manure.
77 However, complicated technical requirements limit implementation of manure composting and
78 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 °C. 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 inactivate 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

102 conducted. The objective of this study was to determine the fate of pirlimycin and ARGs in dairy
103 manure slurries in response to initial pH (5, 7, 9, and 12) shocks and temperatures (10, 35, and
104 55°C) at which the manure is stored. We hypothesized that a simple change of temperature or
105 initial pH would affect the removal of pirlimycin and prevalence of ARGs in dairy manure
106 slurries.

107 **2. Materials and methods**

108 ***2.1. Manure generation***

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

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

129 ***2.2. Manure slurry incubation***

130 Manure slurry was generated by mixing manure and sterile water to achieve a final solid
131 content of 5% to mimic the solid content in a typical manure slurry storage lagoon. Aliquots (200
132 mL) of slurry were transferred into 400 mL glass beakers. The initial manure slurry pH was 7.5.
133 To simulate psychrophilic, mesophilic, and thermophilic environments, beakers were incubated
134 at 10, 35, or 55°C without initial pH adjustment for 90 days. Similarly, slurry samples in beakers
135 were incubated at consistent ambient temperature 25°C for 90 days with initial pH adjusted to 5,
136 7, 9, or 12 using 1 M HCl or NaOH. Each treatment had 4 replicates. Beakers were covered with
137 aluminum foil with a hole in the middle to maintain aerobic conditions, weighed daily, and
138 weight loss due to water evaporation was replaced with sterilized water. Manure slurry was
139 totally mixed using a clean glass rod, and 2 mL was sampled from each beaker on day 0, 1, 3, 7,
140 14, 28, 56, and 90 to monitor pirlimycin concentrations. Samples were stored at -20°C until
141 analysis for pirlimycin. On day 0, 3, 7, and 28 additional 2 mL sample was collected,
142 immediately freeze dried, and stored at -80°C for future DNA extraction.

143 ***2.3. Quantification of pirlimycin***

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

148 (250 mg sorbent, Waters Corp., Milford, MA) fitted in a 20 port SPE vacuum manifold (Agilent
149 Technologies, Lexington, MA). The cleaned-up extract was analyzed for pirlimycin using
150 Agilent 1290 ultra-performance liquid chromatography (UPLC) coupled with Agilent 6490
151 Triple Quad tandem mass spectrometer (Agilent Technologies, Santa Clara, CA).

152 ***2.4.DNA extraction and quantitative Real-Time Polymerase Chain Reaction (q-PCR)***

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

156 Real-time PCR was performed to quantify a macrolide ARG (*mefA*), a tetracycline ARG
157 (*tet(W)*), and a β -lactam ARG (*cfxA*) using an EvaGreen assay with previously published
158 primers (Aminov et al., 2001; Looft et al., 2012; S3ki et al., 2011). These three ARGs were
159 chosen because our prior metagenomic analysis study demonstrated that tetracycline, MLS, and
160 β -lactam antibiotic resistance genes were the most dominant ARGs in feces from the dairy cows
161 treated with pirlimycin, and the pirlimycin administration increased the abundance of these genes
162 compared with the feces from untreated cows (Caudle, 2014), so an effect of temperature or pH
163 on the three ARGs was expected. To normalize the quantities of each ARG to the total bacterial
164 population, the bacterial 16S rRNA gene was quantified using the approach as described by
165 Suzuki et al. (2000). Standard solutions were made from a 10-fold serial dilution of a cloned
166 gene ranging from 10^8 to 10^2 gene copies/ μ l. Based on the standard solutions, standard curves
167 were constructed to quantify gene copies of each ARG. Samples were analyzed in triplicate with
168 a standard curve and a negative control in each run.

169 ***2.5.Statistical analysis***

170 Data processing and analyses were conducted in R software (R development Core Team,
171 2015). Pirlimycin concentration was calculated based on dry manure weight (unit is ng/g). ARGs
172 were normalized to gene copies per bacterial 16S rRNA gene and then natural log transformed.
173 One-way analysis of variance (ANOVA) was carried out to test manure slurry incubation
174 temperature or initial pH effect on pirlimycin concentrations and ARG abundances at each time
175 point using the lm function in R. Multiple paired comparisons were conducted using the Tukey's
176 honestly significant difference (HSD). Significant differences were declared at $P < 0.05$.

177 **3. Results and discussion**

178 ***3.1. Temperature effect on the removal of pirlimycin***

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

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

202 The observed increase in pirlimycin concentration in the early stages (1 to 14 days) of
203 incubation was likely due to deconjugation of pirlimycin conjugates back to pirlimycin. Hornish
204 et al. (1998) indicated that pirlimycin was converted to pirlimycin sulfoxide and pirlimycin
205 sulfone conjugates in bovine liver. A similar deconjugation phenomenon in raw manure-
206 amended soils was reported by Chen et al. (2018). Hornish et al. (1992) demonstrated that
207 approximately 50% of pirlimycin was excreted through milk, and 34% of pirlimycin was
208 released via feces and urine following pirlimycin intramammary administration. In the current
209 study, in total 100 mg pirlimycin was injected into teat canal, and on average 24 kg manure (dry
210 matter based) was totally collected from day 1 to day 4 for each cow. The maximum pirlimycin
211 concentration is 1417 ng/g in the manure mixture assuming 34% elimination rate in manure and
212 no formation of conjugates. The estimated maximum concentration was consistent with the peak
213 pirlimycin concentration at the thermophilic environment on day 1. Based on the initial
214 pirlimycin concentration and the estimated maximum concentration, one could speculate that
215 pirlimycin conjugates accounted for roughly 87% of pirlimycin residues (including pirlimycin

216 and its conjugated forms) in manure. Apparently, the deconjugation of pirlimycin conjugates was
217 predominant in the early stages, and the thermophilic environment facilitated the deconjugation,
218 while the psychrophilic and thermophilic conditions delayed the deconjugation compared to the
219 thermophilic condition. Along with deconjugation of pirlimycin conjugates, pirlimycin and its
220 conjugates can be simultaneously degraded through both abiotic and biotic processes (Aga et al.,
221 2016; Hornish et al., 1992; Ray et al., 2017). Loftin et al. (2008) indicated that lincomycin type
222 antibiotics were rarely hydrolyzed at temperature relevant to environmental conditions because
223 of its recalcitrant linkage structure. However, Hornish et al. (1992) demonstrated that microbes
224 in the digestive tract of dairy cows converted pirlimycin and pirlimycin sulfoxide conjugate to
225 ribonucleotide adducts. Thus, the removal of pirlimycin and its conjugates by fecal microbes
226 likely explains the eventual decreased pirlimycin concentrations across temperature treatments
227 over time.

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

239 **3.2. Initial pH effect on the removal of pirlimycin**

240 Pirlimycin concentrations in response to incubation time at different initial pH with
241 consistent ambient temperature 25 °C are shown in Figure 2. On average, the initial pirlimycin
242 concentration was 171.5 ng/g. As occurred during incubation of manure slurry samples at
243 varying temperature, pirlimycin concentrations increased to a plateau on day 7 at initial pH 9,
244 and on day 28 at initial pH 7 and 12 (at concentrations 5.8, 4.7, and 5.0 times greater than initial,
245 respectively), suggesting that the deconjugation of pirlimycin conjugates was more dominant
246 than removal in the early stages (1 to 28 days). After that, degradation began to
247 surpass deconjugation of pirlimycin conjugates, resulting in a decrease of pirlimycin
248 concentrations until the end of 90-day incubation. Because of the changes of deconjugation and
249 removal over time among pH treatments, pirlimycin concentrations at pH 7 and 9 shocks were
250 greater than that at pH 12 on day 3 and 7 ($P < 0.05$). However, no significant difference of
251 pirlimycin concentration was observed among the neutral and alkaline shocks on day 14, 28, and
252 56. Although pirlimycin concentrations at the alkaline shocks were less than the neutral shock on
253 day 90 ($P < 0.05$), their concentrations were still greater than initial concentrations by 2.1-fold
254 and 1.8-fold, respectively, at pH 9 and 12 shocks. At the acidic shock, the pirlimycin
255 concentrations significantly increased within the first 3 days, resulting in a greater pirlimycin
256 concentration than the other pH treatments on day 3 ($P < 0.05$). Instead of a decrease after
257 reaching a plateau, the pirlimycin concentrations (around 1100 ng/g) remained 6.8 times of the
258 initial level (161.8 ng/g) on day 56 and day 90, which was greater than the rest of pH treatments
259 ($P < 0.05$). The peak pirlimycin concentration under the acidic shock was close to the estimated
260 maximum pirlimycin concentration (1417 ng/n). This suggests that the acidic shock facilitated
261 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 40°C.

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 37°C (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

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

313 ***3.4. Temperature and initial pH effects on the prevalence of *mefA****

314 The *mefA* relative abundance linearly declined over time within each of the temperature
315 treatments (Table 1 and Figure 3). No significant temperature effect on *mefA* abundance was
316 observed on day 3. However, compared to the psychrophilic and mesophilic environments, the
317 thermophilic reactors had decreased *mefA* relative abundance on day 7 and 28 ($P = 0.02$ and
318 0.04). There was no *mefA* abundance difference between the psychrophilic and mesophilic
319 environment on day 7, but on day 28 *mefA* relative abundance was greater at the mesophilic
320 environment than the psychrophilic environment.

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

331 psychrophilic and mesophilic conditions, suggesting that high temperature facilitated the
332 removal of host bacteria that encoded the *mefA* gene.

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

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

350 ***3.5. Temperature and initial pH effects on the prevalence of tet(W)***

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

354 thermophilic environment (Figure 3). On day 3, both the psychrophilic and thermophilic
355 environment exhibited a greater *tet(W)* relative abundance than the mesophilic environment ($P <$
356 0.05; Table 1). However, the *tet(W)* relative abundance at the thermophilic environment was
357 greater than the psychrophilic and mesophilic environment on day 7, and no significant
358 difference was observed among three treatments on day 28.

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

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

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

392 The *cfxA* 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 *cfxA* is the
394 most abundant ARG observed in dairy manure. However, in the current study, the relative
395 abundance of *cfxA* was less than *mefA* 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 *cfxA*
397 abundance, indicating that the bacteria that carrying *cfxA* gene might not be sensitive to these
398 environmental factors or potential pirlimycin selective pressure. However, a decrease of *cfxA*

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

401 **4. Conclusion**

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

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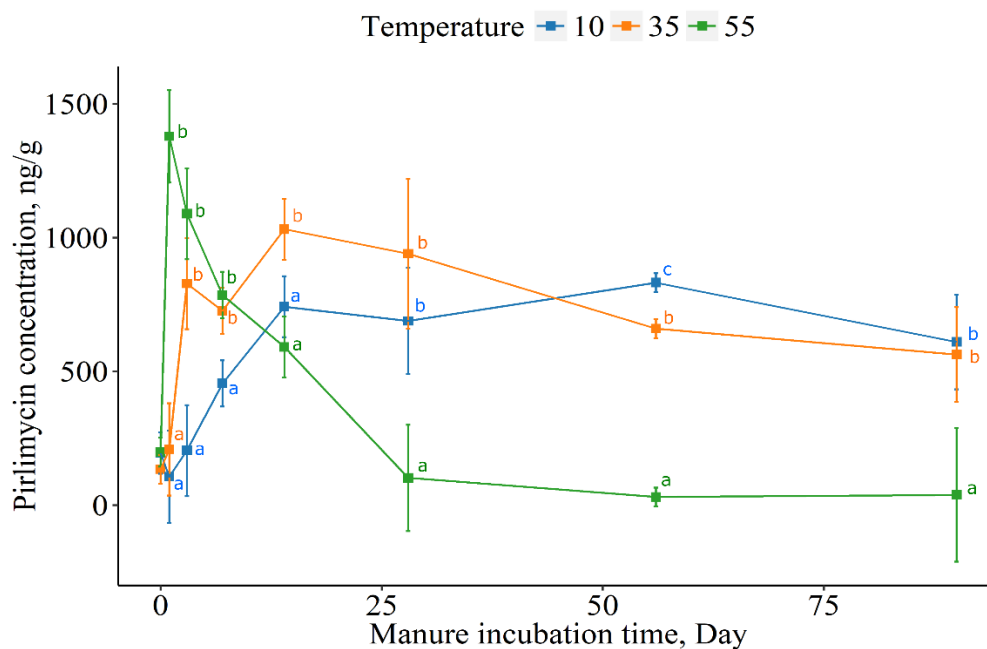
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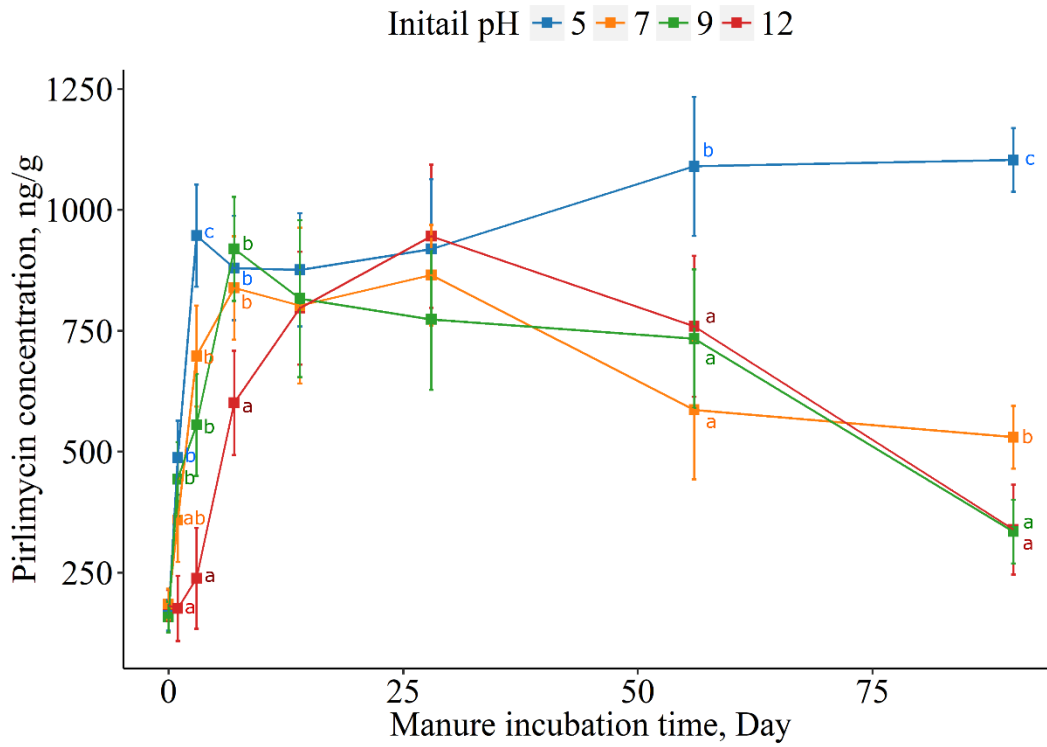
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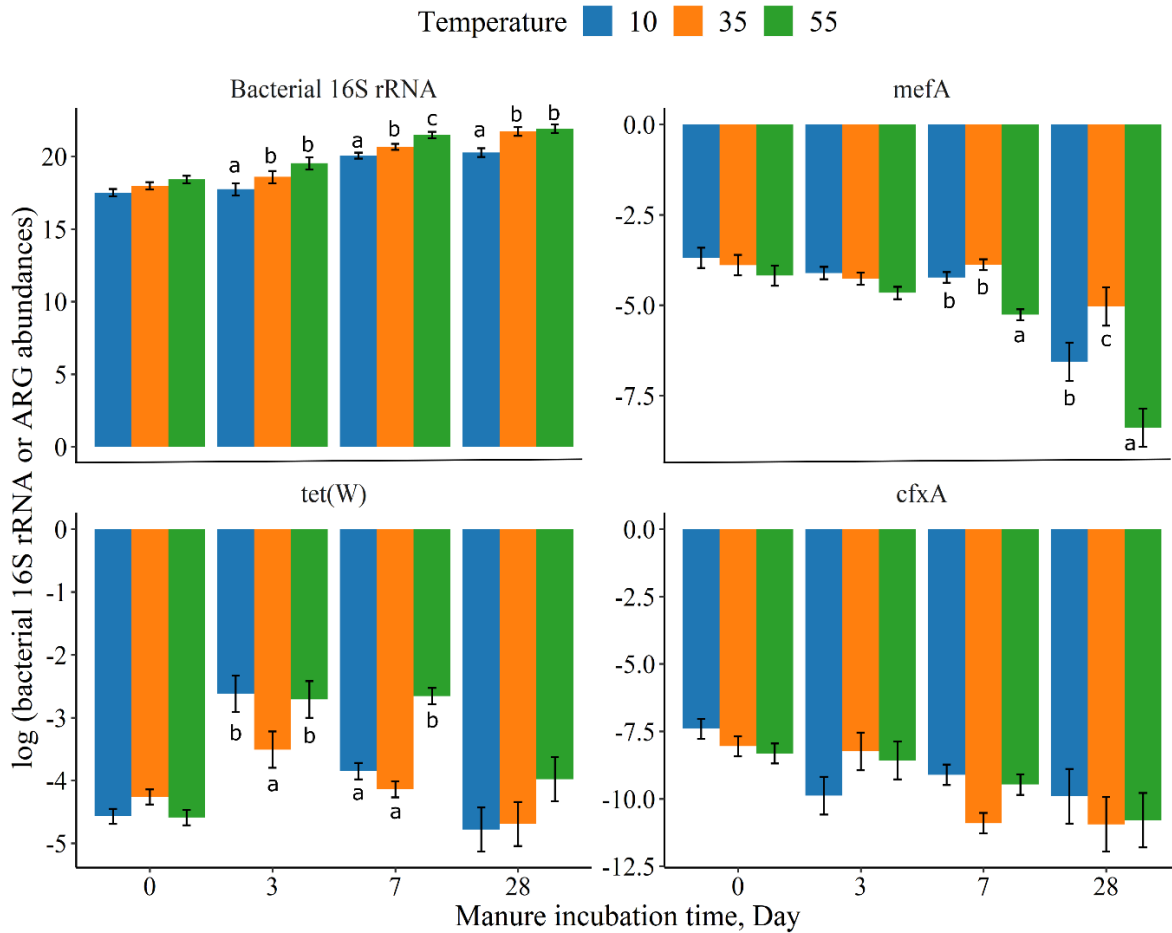


586
 587 Figure 1. Pirlimycin concentrations in dairy manure slurries in response to incubation time at
 588 different temperatures with same initial pH 7.5. The pirlimycin concentration in the slurry on day
 589 0 was 175.8 ng/g. Error bars represent mean of standard error of four replicates (n = 4). Different
 590 characters within the same incubation time indicate significantly different means.



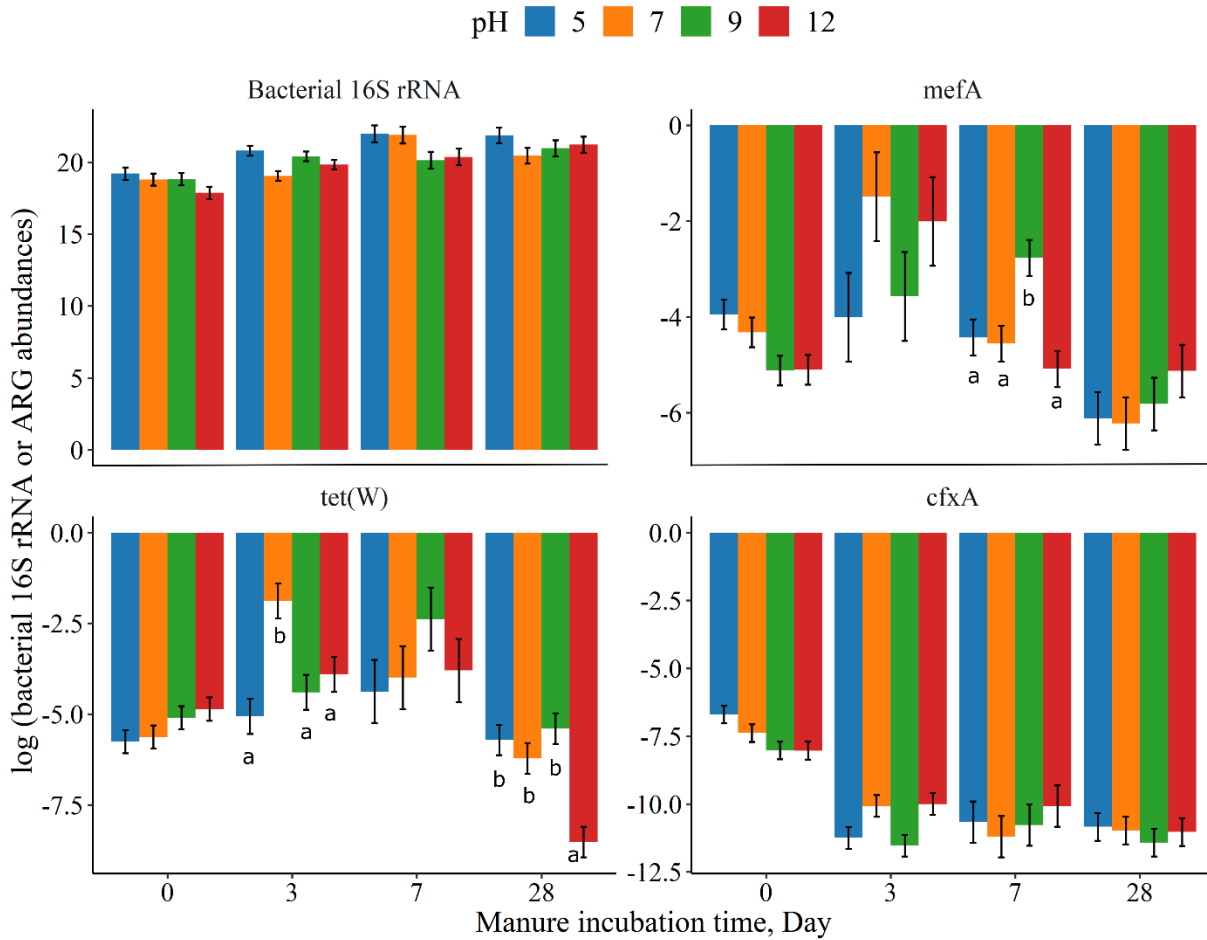
591

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



597

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



605

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

613 Table 1. Effect of temperature on pirlimycin concentrations, bacterial 16S rRNA, and ARG
 614 abundances in dairy manure slurries¹

| Temperature | 10°C | 35°C | 55°C | 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 |
| <i>mefA</i> | | | | | |
| 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 |
| <i>tet(W)</i> | | | | | |
| 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 |
| <i>cfxA</i> | | | | | |
| 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 ¹Bacterial 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 ²Standard error of mean

620 Table 2. Effect of initial pH on pirlimycin concentrations, bacterial 16S rRNA, and ARG
 621 abundances during dairy manure slurries¹

| Initial pH | 5 | 7 | 9 | 12 | SEM ³ | <i>P</i> 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 |
| <i>mefA</i> | | | | | | |
| 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 |
| <i>tet(W)</i> | | | | | | |
| 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 |
| <i>cfxA</i> | | | | | | |
| 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 ¹Bacterial 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 ²Standard error of mean