



# *Increasing temperature and pH can facilitate reductions of cephapirin and antibiotic resistance genes in dairy manure slurries*

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1 INTERPRETIVE SUMMARY: *Li et al* (2019). “*Short communication: Raising temperature and*  
2 *pH can facilitate reductions of cephapirin and antibiotic resistance genes in dairy manure*  
3 *slurries*”. Quantifying the fate of antibiotics and antibiotic resistance genes (ARGs) exposed to  
4 various temperatures and pH adjustments in manure slurries will help guide manure management  
5 practices to minimize the emergence and subsequent spreading of antimicrobial resistance. In the  
6 current study, effects of temperature and initial pH shock on cephapirin and ARGs in dairy  
7 manure slurries were investigated using a microcosm setup. Our results suggest a change in  
8 temperature or initial pH adjustment during storage of dairy manure slurries could mitigate the  
9 spread of resistance.

10 ***Short communication: Raising temperature and pH can facilitate reductions of cephalosporin***  
11 ***and antibiotic resistance genes in dairy manure slurries***

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17 **Abstract**

18 Quantifying antibiotics and antibiotic resistance genes (ARGs) in manure exposed to various  
19 temperature and pH treatments could guide the development of cost-effective manure handling  
20 methods to minimize the spread of antibiotic resistance following land application of manure.  
21 This study aimed to investigate the effect of various temperatures and initial pH shocks on the  
22 persistence of a cephalosporin antibiotic and ARGs in dairy manure slurries. Feces and urine  
23 were collected from five healthy dairy cows administered with cephapirin (cephalosporin  
24 antibiotic) at dry-off via intramammary infusion, and mixed with sterile water to generate  
25 manure slurries. In a 28-day incubation study, dairy manure slurries were either continuously  
26 exposed to one of three temperatures (10, 35, and 55°C) or received various initial pH (5, 7, 9,  
27 and 12) shocks. Cephapirin was detected in the initial samples and on day 1 following all  
28 treatments, but it was undetectable thereafter. This indicates that cephapirin can be rapidly  
29 degraded irrespective of temperature and pH treatments. However, degradation was greater on  
30 day 1 with the mesophilic (35°C) and thermophilic (55°C) environments compared to the  
31 psychrophilic environment at 10°C ( $P < 0.001$ ). Increasing pH beyond neutral also accelerated  
32 degradation as cephapirin concentrations were lower on day 1 after initial alkaline adjustments  
33 (pH 9 and 12) than neutral and acidic adjustments (pH 7 and 5;  $P < 0.001$ ). No significant effect  
34 of temperature or initial pH was observed on abundances of a beta-lactam ARG, *cfxA*, and a  
35 tetracycline ARG, *tet(W)*, implying that bacteria that encoded *cfxA* or *tet(W)* genes were not  
36 sensitive to temperature or pH in dairy manure slurries. However, abundances of a macrolide  
37 ARG, *mefA*, were decreased in the psychrophilic and thermophilic environments, and also  
38 following exposure to a strong alkaline shock (pH 12). Our results suggest that increasing  
39 temperature or pH during storage of dairy manure slurries could be used together with other on-

40 farm practices that are tailored to reduce the transfer of ARGs from manure to the environment  
41 following land application.

42 **Key words:** antibiotic resistance genes; cephalosporin; dairy manure slurry

### 43 **Short Communication**

44 In 2017, approximately 10.93 million kg of antimicrobial drugs were sold in the United  
45 States for use in food-producing animals, and ~50% of that was used in cattle (USFDA, 2018).  
46 Previous studies indicated that up to 90% of administered antibiotics are eliminated from  
47 animals' bodies through feces or urine (Kemper, 2008; Ray et al., 2014), implying that manure  
48 generated from animal production represents a major route of antibiotics transfer to the  
49 environment. The presence of antibiotics, even at a very low concentrations, can contribute to  
50 emergence of antibiotic resistance genes (ARGs) and selection of antibiotic resistant bacteria  
51 (Knapp et al., 2008; Martínez, 2008; Sandegren, 2014). Many studies have demonstrated that  
52 land application of untreated, antibiotic-laden manure can substantially enhance the abundance  
53 of ARGs in agricultural soils (Fahrenfeld et al., 2014; Hu et al., 2016; Gou et al., 2018). These  
54 ARGs can be transferred to animals and humans through drinking water or the food chain,  
55 leading to decreased effectiveness of subsequent antibiotic therapies (Alcaine et al., 2005; Tello  
56 et al., 2012). Therefore, it is vital to develop cost-effective methods to degrade antibiotics in  
57 manure before land application to mitigate the dissemination of antibiotic resistance.

58 Manure management practices such as composting and anaerobic digestion could  
59 effectively increase degradation of antibiotics and reduce the prevalence of ARGs (Qian et al.,  
60 2016; Sun et al., 2016; Ray et al., 2017; Gou et al., 2018). Ray et al. (2017) indicated that  
61 thermophilic temperature during the manure composting process could completely degrade  
62 cephalosporin, lincosamide, and sulfonamide antibiotics. Sun et al. (2016) reported that the

63 abundances of 8 of 10 detected ARGs declined during thermophilic anaerobic digestion of dairy  
64 manure. However, high maintenance and operation costs, as well as high technical requirements,  
65 limit implementation of composting or anaerobic digester operations on many farms  
66 (Pandyaswargo and Premakumara, 2014; Garfí et al., 2016). Physico-chemical factors, such as  
67 temperature, pH, CO<sub>2</sub> and O<sub>2</sub> levels, likely explain the effect of composting and anaerobic  
68 digestion on persistence of antibiotics and ARGs (Sun et al., 2016; Youngquist et al., 2016; Li et  
69 al. 2019). The objective of the current study was to isolate two of these factors, temperature and  
70 pH, and evaluate their independent effects on antibiotic and ARG persistence. Understanding  
71 these mechanisms will inform future development of best management practices for preventing  
72 the spread of antibiotic resistance from livestock farms.

73         Five dairy cows at the end of lactation with similar body weight ( $574.9 \pm 32.8$  kg) were  
74 fitted with urinary catheters to allow separate collection of feces and urine and moved to  
75 individual metabolic stalls at the Virginia Tech Dairy Center (Blacksburg, VA). After 24 h of  
76 acclimation to the barn and catheters, the cows were treated with a single dose of 300 mg  
77 cephapirin benzathine (Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO) into each of the  
78 four mammary glands through the teat as in the standard dry-off protocol (dry cow antibiotic  
79 treatment 60 days before calving) of the Virginia Tech Dairy Center. All cows were allowed ad  
80 libitum access to water and a ration formulated to meet their nutritional needs.

81         Feces and urine were collected from day 1 (the day cephapirin was administered) through  
82 day 3 following treatments, and then all feces and urine excreted by a cow over the three days  
83 were combined to form one homogenous pool of manure. Approximately 1 kg of the manure  
84 mixture was mixed with sterile water to generate a manure slurry with a final solid content of  
85 5%. Aliquots (200 mL) of slurry were transferred into glass beakers (400 mL) and incubated at

86 10, 35, and 55°C or at 25°C with initial pH adjustments to 5, 7, 9, or 12 using 1 M (mol/L) HCl  
87 (for pH 5 and 7) or NaOH (for pH 9 and 12) solution. Each treatment had 4 replicates. Beakers  
88 were covered with aluminum foil with a hole in the middle to maintain aerobic conditions.  
89 Beakers were weighed daily, and sterile water was added to each beaker to replace weight loss  
90 due to water evaporation. Samples were collected on days 0, 1, 3, 7, and 28 and were  
91 immediately stored at -20°C for future analysis.

92 Cephapirin concentrations were quantified following the method developed by Ray et al.  
93 (2014). To quantify ARGs, frozen manure slurries were freeze-dried, and then DNA was  
94 extracted from the freeze-dried manure samples using the QIAamp DNA stool extraction kit  
95 (Qiagen, Valencia, CA) following the manufacturer's instructions. Quantitative PCR (qPCR)  
96 was performed to quantify a beta-lactam ARG, *cfxA*, a macrolide ARG, *mefA*, and a tetracycline  
97 ARG, *tet(W)*, using an EvaGreen assay (Biotium, Hayward, CA) with previously reported  
98 primers (Aminov et al., 2001; S6ki et al., 2011; Looft et al., 2012). 16S rRNA gene copy  
99 numbers were determined using the method by Suzuki et al. (2000). Bacterial gene abundances  
100 were represented as 16S rRNA gene copies per g dry manure, and ARG relative abundances  
101 were expressed as the proportion to 16S rRNA gene abundance.

102 Data analyses were conducted in R software (version 3.3.0; R development Core Team,  
103 2015). All qPCR results were normalized using log base 10 transformation. The temperature or  
104 initial pH effect on cephalosporin concentrations and ARGs was analyzed in a mixed statistical  
105 model with temperature or initial pH as a fixed effect and incubation day as a repeated  
106 measurement using the gls function in the nlme package (Pinheiro et al., 2018). The first-order  
107 autoregressive covariance with heterogeneous variance of time were considered in the repeated



108 measures ANOVA model. The Tukey's honestly significant difference post hoc test was used to  
109 test differences among treatments, and  $P < 0.05$  was considered significant.

110 Cephapirin was present in manure slurries on day 0 (initial samples) and day 1 following  
111 either temperature or initial pH adjustment, but it was undetectable thereafter. On day 0, the  
112 mean concentration of cephapirin was  $12.5 \pm 1.3$  ng per g dry manure; this rapidly decreased to  
113 less than 1 ng per g dry manure on day 1 regardless of temperature or pH treatments (Figure 1).  
114 Ray et al. (2017) reported that cephapirin was only present in day 0 compost samples. Gilbertson  
115 et al. (1990) indicated that another cephalosporin (ceftiofur) was quickly degraded within 8 h in  
116 cattle feces. Thus, our result was consistent with the previous studies, suggesting that cephapirin  
117 has a poor stability in dairy manure slurries. Compared to the psychrophilic environment (10°C),  
118 the mesophilic (35°C) and thermophilic (55°C) environments decreased cephapirin  
119 concentrations on day 1, with no difference between the mesophilic and thermophilic conditions  
120 (Figure 1A).

121 Cephapirin belongs to the  $\beta$ -lactam class of antibiotics which have a basic  $\beta$ -lactam ring  
122 structure for the antibacterial activity. Ray et al. (2017) indicated that cephapirin was unstable at  
123 high temperature and Cha et al. (2006) demonstrated that the  $\beta$ -lactam ring has poor stability in  
124 animal manure and lagoon effluent. Wagner et al. (2011) observed that multiple  $\beta$ -lactamases  
125 were detected in bacteria isolated from the digestive tract of cows. In the current study, the  
126 number of bacterial 16S rRNA gene copies increased in the mesophilic and thermophilic  
127 environments compared to the psychrophilic environment (Table 1), suggesting that elevated  
128 temperature might facilitate the biotic degradation of cephapirin via the secretion of  $\beta$ -lactamases  
129 by an increased population of manure microbes.

130 On day 1, cephapirin concentration was lower in manure exposed to initial alkaline pH  
131 shock (pH 9 and 12) compared to neutral or acid pH shock (pH 7 or 5; Figure 1B). Gilbertson et  
132 al. (1990) demonstrated that the hydrolysis of ceftiofur was dramatically increased when pH was  
133 changed from pH 5 to 9. Ivaska and Nordström (1983) found that cephapirin exhibited a stable  
134 peak on differential pulse polarography at pH 7 to 8.5, implying that the chemical structure of  
135 cephapirin is more stable in a slightly alkaline condition.

136 In the current study, none of the animals had received any antibiotic treatment for at least  
137 280 days before the cephapirin administration, but all three of the ARGs quantified (*cfxA*, *mefA*,  
138 and *tet(W)*) were detected in all of the manure slurry samples. Muurinen et al. (2017) detected 8  
139 common ARGs and mobile genetic elements in stored manure as well as in soil (including  
140 fertilized and unfertilized) and tile drainage water collected from 2 dairy farms, which supports  
141 the co-occurrence of ARGs in various farm environments. It is likely that the detected ARGs  
142 could be transferred to the cows from the dairy farm environment or from other cows in the dairy  
143 herd, and therefore, various modes of ARG transmission deserve further attention and research.

144 Tetracycline resistance genes are the common ARGs observed in dairy manure (Sun et  
145 al., 2016; Zhou et al., 2016). In the current study, the average log base 10 transformed relative  
146 abundances for *tet(W)*, *mefA*, and *cfxA* cross all the temperature and pH treatments were -1.8, -  
147 2.2, and -4.3, respectively. A greater abundance of the *tet(W)* gene was detected than of the *cfxA*  
148 or *mefA* gene. The *cfxA* gene encodes class A  $\beta$ -lactamase, a gene that codes for resistance  
149 specific to cephalosporin antibiotics (García et al., 2008). In the current study, the *cfxA* gene had  
150 a low abundance compared to the abundance of *tet(W)* and *mefA* genes. This could be due to the  
151 rapid dissipation of cephapirin during manure incubation leading to low or no selective pressure  
152 among manure microbes.

153 No significant temperature or initial pH effects on the relative abundance of *cfxA* and  
154 *tet(W)* were observed in dairy manure slurry (Table 1). Sun et al. (2016) reported that there was  
155 no significant temperature effect on *tet(W)* abundance during anaerobic digestion of dairy  
156 manure. Huang et al. (2019) demonstrated that high temperature cannot always remove ARGs in  
157 manure anaerobic digestion unless antibiotic resistant bacteria or gene transfer elements are more  
158 efficiently decreased by raising temperature. Therefore, we can conclude that manure bacteria  
159 that encoded *cfxA* or *tet(W)* genes were not sensitive to incubation temperature or initial pH  
160 shock in dairy manure slurries.

161 Compared to the mesophilic condition, the *mefA* gene abundance decreased in  
162 psychrophilic and thermophilic environments ( $P < 0.0001$ ; Table 1) probably due to changes in  
163 bacteria community (Qian et al., 2016; Sun et al., 2016). Miller et al. (2016) indicated that high  
164 temperature could remove some ARGs carried by bacterial hosts that were not thermotolerant.  
165 The optimum growth temperature for most bacterial species is around 37°C (Zhu, 2000).  
166 Therefore, the psychrophilic and thermophilic environments might inhibit the growth rate of  
167 bacteria that encoded *mefA* gene, leading to the decrease of *mefA* abundance.

168 The strong alkaline treatment (pH 12) resulted in 0.9 log base 10 fold reduction of *mefA*  
169 abundance as compared to the other pH treatments ( $P < 0.0001$ ; Table 1). Although there was no  
170 pH effect on total bacterial 16S rRNA gene copies (Table 1), it is possible that the microbial  
171 community structure shifted due to pH stress. Lin et al. (2013) reported that bacteria *Clostridium*  
172 *alkalicellum* and *Corynebacterium humireducens* were enriched at pH 10 in swine manure, while  
173 the abundance of *Butyricimonas sp.* was decreased. In the current study, the strong alkaline  
174 condition might have changed the bacterial community to have less bacteria carrying *mefA* gene,  
175 which led to the reduction in *mefA* abundance.

176 In conclusion, the thermophilic temperatures and strong alkaline treatments can facilitate  
177 rapid dissipation of cephalosporin. Both psychrophilic and thermophilic temperatures as well as  
178 strong alkaline treatments can decrease the abundance of *mefA* ARG. However, cephalosporin was  
179 rapidly and completely removed from manure slurries regardless of heat and pH treatment and  
180 thus, likely, no additional treatments are needed for its degradation. On the other hand, heat and  
181 pH influenced the abundance of *mefA* gene. This indicates that there could be treatment  
182 strategies (such as change in temperature or initial pH adjustment) developed to aid the removal  
183 of *mefA*. For instance, adding hydrated lime into manure slurries could be an effective method to  
184 reduce antibiotic resistance, since it will generate heat and increase pH. Heinonen-Tanski et al.  
185 (2004) reported that a dose of 10 g/L of hydrated lime with good stirring could destroy coliform  
186 bacteria to concentrations below 10 CFU/g in diluted cattle slurries. Jamal et al. (2011) indicated  
187 that adjusting pH to 12 or higher can halt or retard the microbial reactions leading to decreased  
188 odor production and vector attraction. Meanwhile, high pH can also reduce the availability of  
189 heavy metals, enhancing agricultural benefits and lowering environmental risks (Wong and  
190 Selvam, 2006). However, the role of elevated manure temperature and pH to nitrogen losses via  
191 volatilization of ammonia should be mentioned. The significant volatilization of ammonia during  
192 manure liming is a safety concern for animals and workers, and the loss of N also represents a  
193 significant loss of value for the manure as a fertilizer. Therefore, large stores of manure slurry  
194 should never be treated with lime due to a safety concern and an economic loss. Rather, liming  
195 of manure might be conducted within small loads of manure being hauled to a land application  
196 site in a tank wagon. One potential limitation of this study is that manure samples that had been  
197 treated to adjust pH were not neutralized before freezing. Without neutralizing samples at the  
198 completion of the incubation time, pH might impact the samples during storage and later

199 analysis. Although immediate storage at -20°C would stop almost all microbial growth and  
200 chemical properties of fresh manure (Pan et al., 2019), it may be worth highlighting that -80°C  
201 instead of -20°C is the best storage temperature for microbial community analyses, and pH may  
202 have impacted the degradation of cephapirin. Overall, our results suggest increasing temperature  
203 or pH during storage of dairy manure slurries could be used together with other on-farm  
204 practices that are tailored to reduce the transfer of ARGs from manure to the environment  
205 following land application.

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## 209 **Reference**

210 Alcaine, S. D., S. S. Sukhnanand, L. D. Warnick, W.-L. Su, P. McGann, P. McDonough, and M.  
211 Wiedmann. 2005. Ceftiofur-resistant Salmonella strains isolated from dairy farms represent  
212 multiple widely distributed subtypes that evolved by independent horizontal gene transfer.  
213 *Antimicrob. Agents Chemother.* 49(10):4061-4067.

214 Aminov, R., N. Garrigues-Jeanjean, and R. Mackie. 2001. Molecular ecology of tetracycline  
215 resistance: development and validation of primers for detection of tetracycline resistance genes  
216 encoding ribosomal protection proteins. *Appl. Environ. Microbiol.* 67(1):22-32.

217 Cha, J., S. Yang, and K. Carlson. 2006. Trace determination of  $\beta$ -lactam antibiotics in surface  
218 water and urban wastewater using liquid chromatography combined with electrospray tandem  
219 mass spectrometry. *J. Chromatogr. A* 1115(1-2):46-57.

220 Fahrenfeld, N., K. Knowlton, L. A. Krometis, W. C. Hession, K. Xia, E. Lipscomb, K. Libuit, B.  
221 L. Green, and A. Pruden. 2014. Effect of manure application on abundance of antibiotic

222 resistance genes and their attenuation rates in soil: field-scale mass balance approach. *Environ.*  
223 *Sci. Technol.* 48(5):2643-2650.

224 García, N., G. Gutiérrez, M. Lorenzo, J. E. García, S. Píriz, and A. Quesada. 2008. Genetic  
225 determinants for *cfxA* expression in *Bacteroides* strains isolated from human infections. *J.*  
226 *Antimicrob. Chemother.* 62(5):942-947.

227 Garfí, M., J. Martí-Herrero, A. Garwood, and I. Ferrer. 2016. Household anaerobic digesters for  
228 biogas production in Latin America: A review. *Renew. Sust. Energ. Rev.* 60:599-614.

229 Gilbertson, T. J., R. E. Hornish, P. S. Jaglan, K. T. Koshy, J. L. Nappier, G. L. Stahl, A. R.  
230 Cazars, J. M. Nappier, and M. F. Kubicek. 1990. Environmental fate of ceftiofur sodium, a  
231 cephalosporin antibiotic. Role of animal excreta in its decomposition. *J. Agric. Food Chem.*  
232 38(3):890-894.

233 Gou, M., H.-W. Hu, Y.-J. Zhang, J.-T. Wang, H. Hayden, Y.-Q. Tang, and J.-Z. He. 2018.  
234 Aerobic composting reduces antibiotic resistance genes in cattle manure and the resistome  
235 dissemination in agricultural soils. *Sci. Total Environ.* 612:1300-1310.

236 Heinonen-Tanski, H., S. Antola, and K. Weppling. 2004. Hydrated lime and Velox rapidly  
237 reduce enteric microorganisms in manure. *Sustainable Organic Waste Management for*  
238 *Environmental Protection and Food Safety. RAMRAN 2004, FAO and CSIC*, 2:33-36.

239 Hu, H. W., J. T. Wang, J. Li, J. J. Li, Y. B. Ma, D. Chen, and J. Z. He. 2016. Field-based  
240 evidence for copper contamination induced changes of antibiotic resistance in agricultural soils.  
241 *Environ. Microbiol.* 18(11):3896-3909.

242 Huang, X., J. Zheng, S. Tian, C. Liu, L. Liu, L. Wei, H. Fan, T. Zhang, L. Wang, and G. Zhu.  
243 2019. Higher Temperatures Do Not Always Achieve Better Antibiotic Resistance Gene Removal  
244 in Anaerobic Digestion of Swine Manure. *Appl. Environ. Microbiol.* 85(7):e02878-02818.

245 Ivaska, A. and F. Nordström. 1983. Determination of some cephalosporins by differential pulse  
246 polarography and linear scan voltammetry. *Anal. Chim. Acta* 146:87-95.

247 Jamal, A., N. Norieh, and M. Farzadkia. 2011. Comparison of aerobic and lime stabilization  
248 methods for evaluation of sewage sludge reuse. *J. Environ. Sci. Technol.* 4(2):182-190.

249 Kemper, N. 2008. Veterinary antibiotics in the aquatic and terrestrial environment. *Ecol. Indic.*  
250 8(1):1-13.

251 Knapp, C. W., C. A. Engemann, M. L. Hanson, P. L. Keen, K. J. Hall, and D. W. Graham. 2008.  
252 Indirect evidence of transposon-mediated selection of antibiotic resistance genes in aquatic  
253 systems at low-level oxytetracycline exposures. *Environ. Sci. Technol.* 42(14):5348-5353.

254 Li, R., J. A. Jay, and M. K. Stenstrom. 2019. Fate of antibiotic resistance genes and antibiotic-  
255 resistant bacteria in water resource recovery facilities. *Water Environ. Res.* 91(1):5-20.

256 Lin, L., C. Wan, X. Liu, D.-J. Lee, Z. Lei, Y. Zhang, and J. H. Tay. 2013. Effect of initial pH on  
257 mesophilic hydrolysis and acidification of swine manure. *Bioresour. Technol.* 136:302-308.

258 Looft, T., T. A. Johnson, H. K. Allen, D. O. Bayles, D. P. Alt, R. D. Stedtfeld, W. J. Sul, T. M.  
259 Stedtfeld, B. Chai, and J. R. Cole. 2012. In-feed antibiotic effects on the swine intestinal  
260 microbiome. *Proc. Natl. Acad. Sci.* 109(5):1691-1696.

261 Martínez, J. L. 2008. Antibiotics and antibiotic resistance genes in natural environments. *Science*  
262 321(5887):365-367.

263 Miller, J. H., J. T. Novak, W. R. Knocke, and A. Pruden. 2016. Survival of antibiotic resistant  
264 bacteria and horizontal gene transfer control antibiotic resistance gene content in anaerobic  
265 digesters. *Front. Microbiol.* 7:263.

266 Muurinen, J., R. Stedtfeld, A. Karkman, K. Pärnänen, J. Tiedje, and M. Virta. 2017. Influence of  
267 manure application on the environmental resistome under Finnish agricultural practice with  
268 restricted antibiotic use. *Environ. Sci. Technol.* 51(11):5989-5999.

269 Pan, J., J. Fadel, R. Zhang, H. El-Mashad, Y. Ying, and T. Rumsey. 2009. Evaluation of sample  
270 preservation methods for poultry manure. *Poult. Sci.* 88(8):1528-1535.

271 Pandyaswargo, A. H. and D. G. J. Premakumara. 2014. Financial sustainability of modern  
272 composting: the economically optimal scale for municipal waste composting plant in developing  
273 Asia. *Int. J. Recycl. Org. Waste Agric.* 3(3):4.

274 Pinheiro, J., D. Bates, S. DebRoy, D. Sarkar, and R. C. Team. 2018. nlme: Linear and nonlinear  
275 mixed effects models. R package version version 3.1-137.

276 Qian, X., W. Sun, J. Gu, X.-J. Wang, Y.-J. Zhang, M.-L. Duan, H.-C. Li, and R.-R. Zhang. 2016.  
277 Reducing antibiotic resistance genes, integrons, and pathogens in dairy manure by continuous  
278 thermophilic composting. *Bioresour. Technol.* 220:425-432.

279 R development Core Team. 2015. R: A language and environment for statistical computing.  
280 <http://www.R-project.org>.

281 Ray, P., C. Chen, K. F. Knowlton, A. Pruden, and K. Xia. 2017. Fate and effect of antibiotics in  
282 beef and dairy manure during static and turned composting. *J. Environ. Qual.* 46(1):45-54.

283 Ray, P., K. F. Knowlton, C. Shang, and K. Xia. 2014. Development and validation of a UPLC-  
284 MS/MS method to monitor cephapirin excretion in dairy cows following intramammary infusion.  
285 *PloS one* 9(11):e112343.

286 Sandegren, L. 2014. Selection of antibiotic resistance at very low antibiotic concentrations. *Ups.*  
287 *J. Med. Sci.* 119(2):103-107.



288 SÓki, J., S. M. Gonzalez, E. Urbán, E. Nagy, and J. A. Ayala. 2011. Molecular analysis of the  
289 effector mechanisms of cefoxitin resistance among *Bacteroides* strains. *J. Antimicrob.*  
290 *Chemother.* 66(11):2492-2500.

291 Sun, W., X. Qian, J. Gu, X.-J. Wang, and M.-L. Duan. 2016. Mechanism and effect of  
292 temperature on variations in antibiotic resistance genes during anaerobic digestion of dairy  
293 manure. *Sci. Rep.* 6:30237.

294 Suzuki, M. T., L. T. Taylor, and E. F. DeLong. 2000. Quantitative analysis of small-subunit  
295 rRNA genes in mixed microbial populations via 5'-nuclease assays. *Appl. Environ. Microbiol.*  
296 66(11):4605-4614.

297 Tello, A., B. Austin, and T. C. Telfer. 2012. Selective pressure of antibiotic pollution on bacteria  
298 of importance to public health. *Environ. Health Perspect.* 120(8):1100-1106.

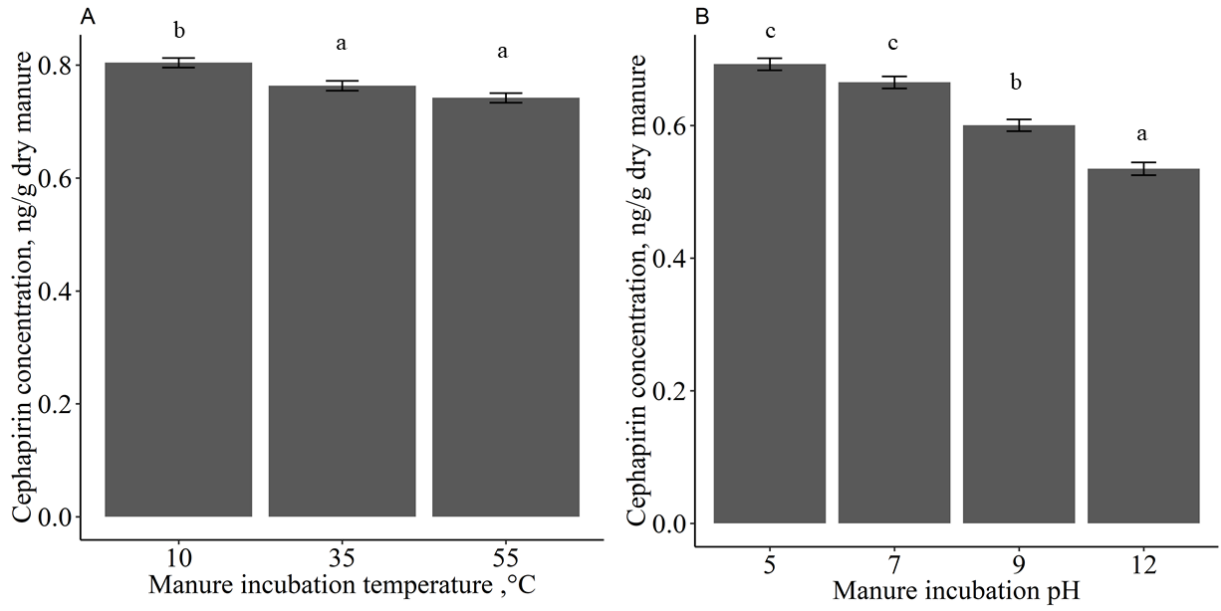
299 USFDA. 2018. 2017 summary report on antimicrobials sold or distributed for use in food-  
300 producing animals.  
301 [https://www.fda.gov/downloads/ForIndustry/UserFees/AnimalDrugUserFeeActADUFA/UCM62](https://www.fda.gov/downloads/ForIndustry/UserFees/AnimalDrugUserFeeActADUFA/UCM628538.pdf)  
302 [8538.pdf](https://www.fda.gov/downloads/ForIndustry/UserFees/AnimalDrugUserFeeActADUFA/UCM628538.pdf).

303 Wagner, R. D., S. J. Johnson, C. E. Cerniglia, and B. D. Erickson. 2011. Bovine intestinal  
304 bacteria inactivate and degrade ceftiofur and ceftriaxone with multiple  $\beta$ -lactamases. *Antimicrob.*  
305 *Agents Chemother.* 55(11):4990-4998.

306 Wong, J.W.C. and A. Selvam, 2006. Speciation of heavy metals during co-composting of sewage  
307 sludge with lime. *Chemosphere*, 63: 980-986.

308 Youngquist, C. P., S. M. Mitchell, and C. G. Cogger. 2016. Fate of antibiotics and antibiotic  
309 resistance during digestion and composting: a review. *J. Environ. Qual.* 45(2):537-545.

- 310 Zhou, B., C. Wang, Q. Zhao, Y. Wang, M. Huo, J. Wang, and S. Wang. 2016. Prevalence and  
311 dissemination of antibiotic resistance genes and coselection of heavy metals in Chinese dairy  
312 farms. *J. Hazard. Mater.* 320:10-17.
- 313 Zhu, J. 2000. A review of microbiology in swine manure odor control. *Agric., Ecosyst. Environ.*  
314 78(2):93-106.



315

316 Figure 1. Cephapirin concentrations in dairy manure slurries exposed to various temperatures (A)

317 or initial pH shocks (B) on day 1 following treatments (n = 4). Bars denoted by a different letter

318 indicate significant differences between treatments ( $P < 0.05$ ).

319 Table 1. The effect of temperature or initial pH on the abundance of 16S rRNA gene and relative  
 320 abundance of antibiotic resistance genes in dairy manure slurries<sup>1</sup>

Treatment	Bacterial 16S rRNA	<i>cfxA</i>	<i>mefA</i>	<i>tet(W)</i>
Temperature, °C				
10	8.28 <sup>a</sup>	-4.03	-2.40 <sup>a</sup>	-1.68
35	8.77 <sup>b</sup>	-4.19	-1.70 <sup>b</sup>	-1.80
55	8.64 <sup>b</sup>	-4.22	-2.69 <sup>a</sup>	-1.65
SEM <sup>2</sup>	0.07	0.08	0.22	0.06
<i>P</i> value for temperature	< 0.0001	0.12	< 0.0001	0.19
Initial pH				
5	8.72	-4.30	-1.97 <sup>b</sup>	-1.94
7	8.75	-4.51	-1.92 <sup>b</sup>	-1.89
9	8.51	-4.32	-1.84 <sup>b</sup>	-1.58
12	8.69	-4.82	-2.81 <sup>a</sup>	-1.99
SEM	0.16	0.22	0.10	0.17
<i>P</i> value for initial pH	0.17	0.3	< 0.0001	0.25

321 <sup>1</sup>Bacterial 16S rRNA gene was expressed as gene copies per g dry manure slurry. *cfxA* is a beta-  
 322 lactam antibiotic resistance gene; *mefA* is a macrolide antibiotic resistance gene; and *tet(W)* is a  
 323 tetracycline antibiotic resistance gene. Antibiotic resistance genes were expressed as a proportion  
 324 to bacterial 16S rRNA gene. Data was normalized using log base 10 transformation. The Tukey's  
 325 post hoc test was used to test differences among treatments. Different superscripts in the same  
 326 column within temperature or pH group indicate significantly different means (*P* < 0.05). n = 4.

327 <sup>2</sup>Standard error of mean