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

Article

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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 cephapirin**

11 **and antibiotic resistance genes in dairy manure slurries**

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**Abstract**

Quantifying antibiotics and antibiotic resistance genes (ARGs) in manure exposed to various

temperature and pH treatments could guide the development of cost-effective manure handling

methods to minimize the spread of antibiotic resistance following land application of manure.

This study aimed to investigate the effect of various temperatures and initial pH shocks on the

persistence of a cephalosporin antibiotic and ARGs in dairy manure slurries. Feces and urine

were collected from five healthy dairy cows administered with cephapirin (cephalosporin

antibiotic) at dry-off via intramammary infusion, and mixed with sterile water to generate

manure slurries. In a 28-day incubation study, dairy manure slurries were either continuously

exposed to one of three temperatures (10, 35, and 55ºC) or received various initial pH (5, 7, 9,

and 12) shocks. Cephapirin was detected in the initial samples and on day 1 following all

treatments, but it was undetectable thereafter. This indicates that cephapirin can be rapidly

degraded irrespective of temperature and pH treatments. However, degradation was greater on

day 1 with the mesophilic (35ºC) and thermophilic (55ºC) environments compared to the

psychrophilic environment at 10ºC ($P < 0.001$). Increasing pH beyond neutral also accelerated

degradation as cephapirin concentrations were lower on day 1 after initial alkaline adjustments

(pH 9 and 12) than neutral and acidic adjustments (pH 7 and 5; $P < 0.001$). No significant effect

of temperature or initial pH was observed on abundances of a beta-lactam ARG, *cfx*A, and a

tetracycline ARG, *tet*(W), implying that bacteria that encoded *cfx*A or *tet*(W) genes were not

sensitive to temperature or pH in dairy manure slurries. However, abundances of a macrolide

ARG, *mef*A, were decreased in the psychrophilic and thermophilic environments, and also

following exposure to a strong alkaline shock (pH 12). Our results suggest that increasing

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; cephapirin; 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

abundances of 8 of 10 detected ARGs declined during thermophilic anaerobic digestion of dairy manure. However, high maintenance and operation costs, as well as high technical requirements, limit implementation of composting or anaerobic digester operations on many farms (Pandyaswargo and Premakumara, 2014; Garfí et al., 2016). Physico-chemical factors, such as temperature, pH, $CO_2$ and $O_2$ levels, likely explain the effect of composting and anaerobic digestion on persistence of antibiotics and ARGs (Sun et al., 2016; Youngquist et al., 2016; Li et al. 2019). The objective of the current study was to isolate two of these factors, temperature and pH, and evaluate their independent effects on antibiotic and ARG persistence. Understanding these mechanisms will inform future development of best management practices for preventing the spread of antibiotic resistance from livestock farms.

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

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

86  10, 35, and 55ºC or at 25ºC 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 -20ºC 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, *cfx*A, a macrolide ARG, *mef*A, and a tetracycline

97  ARG, *tet*(W), using an EvaGreen assay (Biotium, Hayward, CA) with previously reported

98  primers (Aminov et al., 2001; Sóki 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 cephapirin 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 (10ºC),

118    the mesophilic (35ºC) and thermophilic (55ºC) 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 β-lactam class of antibiotics which have a basic β-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 β-lactam ring has poor stability in

124    animal manure and lagoon effluent. Wagner et al. (2011) observed that multiple β-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 β-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 (*cfx*A, *mef*A,

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), *mef*A, and *cfx*A 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 *cfx*A

148     or *mef*A gene. The *cfx*A gene encodes class A β-lactamase, a gene that codes for resistance

149     specific to cephalosporin antibiotics (García et al., 2008). In the current study, the *cfx*A gene had

150     a low abundance compared to the abundance of *tet*(W) and *mef*A 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 *cfx*A 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 *cfx*A 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 *mef*A 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℃ (Zhu, 2000).

166    Therefore, the psychrophilic and thermophilic environments might inhibit the growth rate of

167    bacteria that encoded *mef*A gene, leading to the decrease of *mef*A abundance.

168          The strong alkaline treatment (pH 12) resulted in 0.9 log base 10 fold reduction of *mef*A

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 *mef*A gene,

175    which led to the reduction in *mef*A abundance.

176        In conclusion, the thermophilic temperatures and strong alkaline treatments can facilitate

177    rapid dissipation of cephapirin. Both psychrophilic and thermophilic temperatures as well as

178    strong alkaline treatments can decrease the abundance of *mef*A ARG. However, cephapirin 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 *mef*A 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 *mef*A. 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 -20ºC would stop almost all microbial growth and

200 chemical properties of fresh manure (Pan et al., 2019), it may be worth highlighting that -80ºC

201 instead of -20ºC 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.

206 **Acknowledgements**

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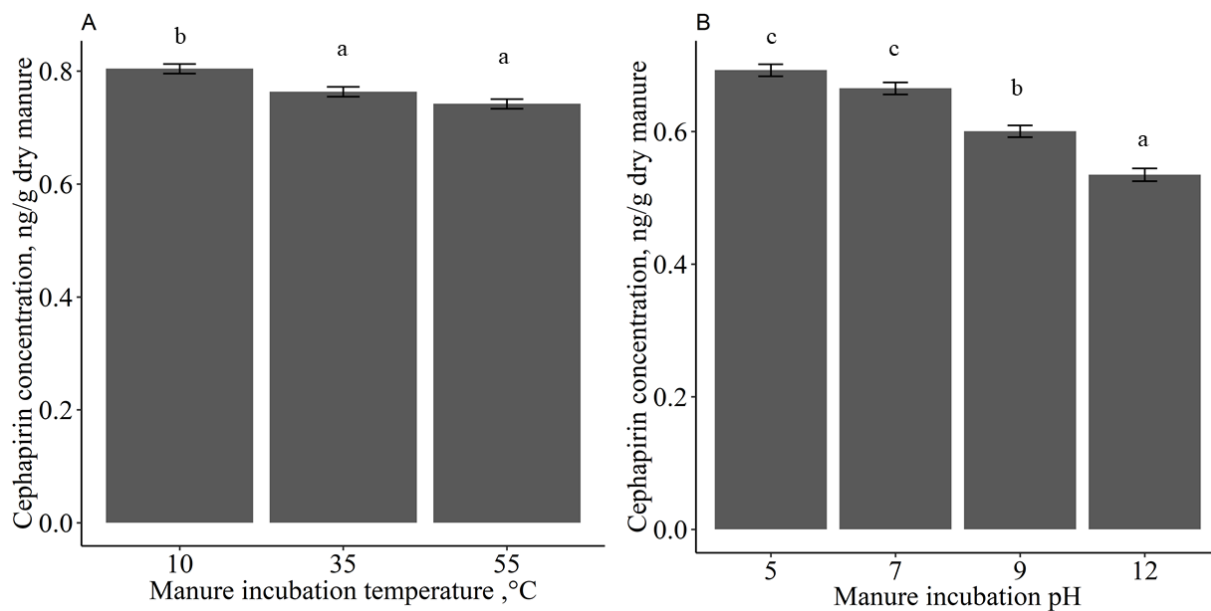
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Figure 1. Cephapirin concentrations in dairy manure slurries exposed to various temperatures (A) or initial pH shocks (B) on day 1 following treatments (n = 4). Bars denoted by a different letter indicate significant differences between treatments ($P < 0.05$).

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[1]

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

321 [1]Bacterial 16S rRNA gene was expressed as gene copies per g dry manure slurry. *cfx*A is a beta-

322 lactam antibiotic resistance gene; *mef*A 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 [2]Standard error of mean