J. Dairy Sci. 103:2877–2882
https://doi.org/10.3168/jds.2019-17453

Short communication: Increasing temperature and pH can facilitate reductions of cephapirin and antibiotic resistance genes in dairy manure slurries

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ABSTRACT

Quantifying antibiotics and antibiotic resistance genes (ARG) 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 ARG in dairy manure slurries. Feces and urine were collected from 5 healthy dairy cows administered with cephapirin (cephalosporin antibiotic) at dry-off via intramammary infusion and were mixed with sterile water to generate manure slurries. In a 28-d incubation study, dairy manure slurries either were continuously exposed to 1 of 3 temperatures (10, 35, and 55°C) or received various initial pH (5, 7, 9, and 12) shocks. Cephapirin was detected in the initial samples and on d 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 d 1 with the mesophilic (35°C) and thermophilic (55°C) environments compared with the psychrophilic environment (10°C). Increasing pH beyond neutral also accelerated degradation as cephapirin concentrations were lower on d 1 after initial alkaline adjustments (pH 9 and 12) than after neutral and acidic adjustments (pH 7 and 5). No significant effect of temperature or initial pH was observed on abundances of a β-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-farm practices that are tailored to reduce the transfer of ARG from manure to the environment following land application.
Key words: antibiotic resistance gene, cephapirin, dairy manure slurry

Short Communication

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

Manure management practices such as composting and anaerobic digestion could effectively increase

Received August 14, 2019.
Accepted November 19, 2019.
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degradation of antibiotics and reduce the prevalence of ARG (Qian et al., 2016; Sun et al., 2016; Ray et al., 2017; Gou et al., 2018). Ray et al. (2017) indicated that thermophilic temperature during the manure composting process could completely degrade cephalosporin, lincosamide, and sulfonamide antibiotics. Sun et al. (2016) reported that the abundances of 8 of 10 detected ARG 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). Physicochemical factors, such as temperature, pH, and CO_2 and O_2 levels, likely explain the effect of composting and anaerobic digestion on persistence of antibiotics and ARG (Sun et al., 2016; Youngquist et al., 2016; Li et al., 2019). The objective of the current study was to isolate 2 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 BW (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 300-mg dose of cephapirin benzathine (Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO) into each of the 4 mammary glands through the teat as in the standard dry-off protocol (dry cow antibiotic treatment 60 d 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 d 1 (the day cephapirin was administered) through d 3 following treatments, and then all feces and urine excreted by a cow over the 3 d were combined to form one homogeneous 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 10, 35, and 55°C or at 25°C with initial pH adjustments to 5, 7, 9, or 12 using 1 *M* HCl (for pH 5 and 7) or NaOH (for pH 9 and 12) solution. Each treatment had 4 replicates. Beakers were covered with aluminum foil with a hole in the middle to maintain aerobic conditions. Beakers were weighed daily, and sterile water was added to each beaker to replace weight loss due to water evaporation. Samples were collected on d 0, 1, 3, 7, and 28 and were immediately stored at −20°C for future analysis.

Cephapirin concentrations were quantified following the method developed by Ray et al. (2014). To quantify ARG, frozen manure slurries were freeze-dried, and then DNA was extracted from the freeze-dried manure samples using the QIAamp DNA stool extraction kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Quantitative PCR was performed to quantify a β-lactam ARG, *cfx*A, a macrolide ARG, *mef*A, and a tetracycline ARG, *tet*(W), using an EvaGreen assay (Biotium, Hayward, CA) with previously reported primers (Aminov et al., 2001; Sóki et al., 2011; Looft et al., 2012). The 16S rRNA gene copy numbers were determined using the method by Suzuki et al. (2000). Bacterial gene abundances were represented as 16S rRNA gene copies per gram of dry manure, and ARG relative abundances were expressed as the proportion to 16S rRNA gene abundance.

Data analyses were conducted in R software (version 3.3.0; R Development Core Team, 2015). All quantitative PCR results were normalized using log base 10 transformation. The effects of temperature or initial pH on cephapirin concentrations and ARG were analyzed in a mixed statistical model with temperature or initial pH as a fixed effect and incubation day as a repeated measurement using the gls function in the nlme package (Pinheiro et al., 2018). The first-order autoregressive covariance with heterogeneous variance of time was considered in the repeated measures ANOVA model. Tukey's honestly significant difference post hoc test was used to test differences among treatments, and $P < 0.05$ was considered significant.

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

Cephapirin belongs to the β-lactam class of antibiotics, which have a basic β-lactam ring structure for

the antibacterial activity. Ray et al. (2017) indicated that cephapirin was unstable at high temperature, and Cha et al. (2006) demonstrated that the β-lactam ring has poor stability in animal manure and lagoon effluent. Wagner et al. (2011) observed that multiple β-lactamases were detected in bacteria isolated from the digestive tract of cows. In the current study, the number of bacterial 16S rRNA gene copies increased in the mesophilic and thermophilic environments compared with the psychrophilic environment (Table 1), suggesting that elevated temperature might facilitate the biotic degradation of cephapirin via the secretion of β-lactamases by an increased population of manure microbes.

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

In the current study, none of the animals had received any antibiotic treatment for at least 280 d before the cephapirin administration, but all 3 of the ARG quantified—*cfx*A, *mef*A, and *tet*(W)—were detected in all of the manure slurry samples. Muurinen et al. (2017) detected 8 common ARG and mobile genetic elements in stored manure as well as in soil (including fertilized and unfertilized) and tile drainage water collected from 2 dairy farms, which supports the co-occurrence of ARG in various farm environments. It is likely that the detected ARG could be transferred to the cows from the dairy farm environment or from other cows in the dairy herd; therefore, various modes of ARG transmission deserve further attention and research.

Tetracycline resistance genes are the common ARG observed in dairy manure (Sun et al., 2016; Zhou et al., 2016). In the current study, the average log base

Table 1. The effect of temperature or initial pH on the abundance of 16S rRNA gene and the relative abundance of antibiotic resistance genes in dairy manure slurries[1]

Item	Bacterial 16S rRNA	*cfx*A	*mef*A	*tet*(W)
Temperature, °C				
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	0.07	0.08	0.22	0.06
P-value	<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	0.17	0.3	<0.0001	0.25

[a,b]Different superscripts in the same column within temperature or pH group indicate significantly different means ($P < 0.05$).

[1]Bacterial 16S rRNA gene was expressed as gene copies per gram of dry manure slurry. *cfx*A is a β-lactam antibiotic resistance gene; *mef*A is a macrolide antibiotic resistance gene; and *tet*(W) is a tetracycline antibiotic resistance gene. Antibiotic resistance genes were expressed as a proportion to bacterial 16S rRNA gene. Data were normalized using log base 10 transformation. Tukey's post hoc test was used to test differences among treatments.

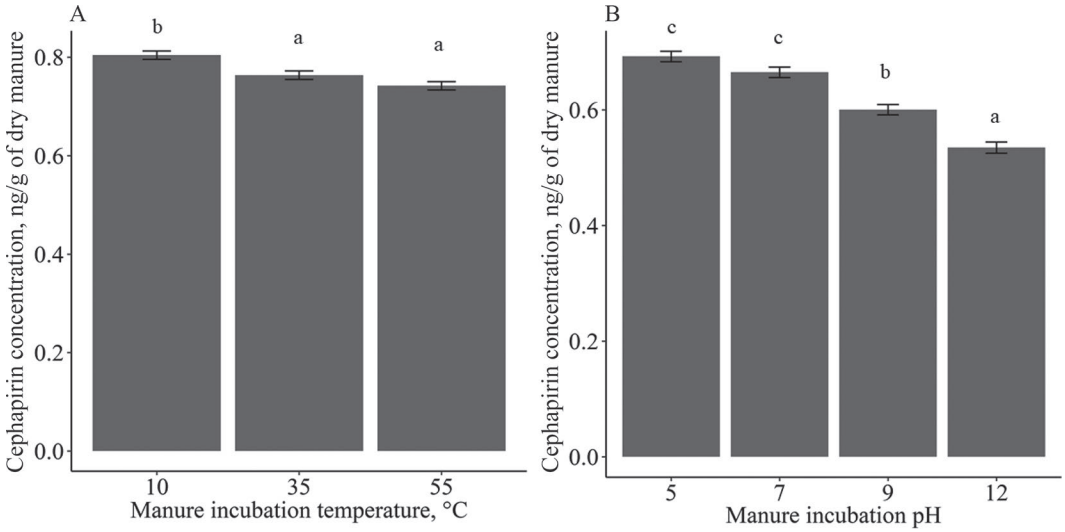

Figure 1. Cephapirin concentrations in dairy manure slurries exposed to various temperatures (A) or initial pH shocks (B) on d 1 following treatments (n = 4). Different letters (a–c) indicate significant differences between treatments ($P < 0.05$). Data are presented as mean ± SEM.

10-transformed relative abundances for *tet*(W), *mef*A, and *cfx*A across all of the temperature and pH treatments were −1.8, −2.2, and −4.3, respectively. A greater abundance of the *tet*(W) gene was detected compared with the *cfx*A or *mef*A genes. The *cfx*A gene encodes class A β-lactamase, a gene that codes for resistance specific to cephalosporin antibiotics (García et al., 2008). In the current study, the *cfx*A gene had a low abundance compared with the abundance of *tet*(W) and *mef*A genes. This could be due to the rapid dissipation of cephapirin during manure incubation leading to low or no selective pressure among manure microbes.

No significant temperature or initial pH effects on the relative abundance of *cfx*A and *tet*(W) were observed in dairy manure slurry (Table 1). Sun et al. (2016) reported that temperature had no significant effect on *tet*(W) abundance during anaerobic digestion of dairy manure. Huang et al. (2019) demonstrated that high temperature cannot always remove ARG in manure anaerobic digestion unless antibiotic-resistant bacteria or gene transfer elements are more efficiently decreased by increasing temperature. Therefore, we can conclude that manure bacteria that encoded *cfx*A or *tet*(W) genes were not sensitive to incubation temperature or initial pH shock in dairy manure slurries.

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

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

In conclusion, the thermophilic temperatures and strong alkaline treatments can facilitate rapid dissipation of cephapirin. Both psychrophilic and thermophilic temperatures as well as strong alkaline treatments can decrease the abundance of *mef*A ARG. However, cephapirin was rapidly and completely removed from manure slurries regardless of heat and pH treatment; thus, it is likely that no additional treatments are needed for its degradation. On the other hand, heat and pH influenced the abundance of the *mef*A gene. This indicates that treatment strategies (e.g., change in temperature or initial pH adjustment) could be developed to aid the removal of *mef*A. For instance, adding hydrated lime into manure slurries could be an effective method to reduce antibiotic resistance because it will generate heat and increase pH. Heinonen-Tanski et al. (2004) reported that a dose of 10 g of hydrated lime/L with good stirring could destroy coliform bacteria to concentrations below 10 cfu/g in diluted cattle slurries. Jamal et al. (2011) indicated that adjusting pH to 12 or higher can halt or slow the microbial reactions, leading to decreased odor production and vector attraction. Meanwhile, high pH can also reduce the availability of heavy metals, enhancing agricultural benefits and lowering environmental risks (Wong and Selvam, 2006). However, the role of elevated manure temperature and pH to nitrogen losses via volatilization of ammonia should be mentioned. The significant volatilization of ammonia during manure liming is a safety concern for animals and workers, and the loss of N represents a significant loss of value for the manure as a fertilizer. Therefore, large stores of manure slurry should never be treated with lime due to safety concerns and economic loss. Rather, liming of manure might be conducted within small loads of manure being hauled to a land application site in a tank wagon. One potential limitation of this study is that manure samples that had been treated to adjust pH were not neutralized before freezing. Without neutralizing samples at the completion of the incubation time, pH might affect the samples during storage and later analysis. Although immediate storage at −20°C would stop almost all microbial growth and chemical properties of fresh manure (Pan et al., 2009), it may be worth highlighting that −80°C instead of −20°C is the best storage temperature for microbial community analyses, and pH may have affected the degradation of cephapirin. Overall, our results suggest that increasing temperature or pH during storage of dairy manure slurries could be used together with other on-farm practices that are tailored to reduce the transfer of ARG from manure to the environment following land application.

ACKNOWLEDGMENTS

This study was supported by NIFA Competitive Grant no. 2014-05280 from the USDA National Insti-

tute of Food and Agriculture (Washington, DC). The authors declare that there is no conflict of interest.

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