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Effect of Dairy Manure Storage Conditions on the Survival of *E. coli* O157:H7 and *Listeria*

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Abstract

Dairy manure is regularly applied to crop fields as a solid or liquid to improve the soil nutrient status. However, pathogens may survive during manure storage and enter the environment during application. In this study, three storage practices were evaluated to understand the survival patterns of *Eschericia coli* O157:H7 and *Listeria* spp. in dairy manure using a culture-based approach. To replicate common farm manure storage techniques, solid manure was stacked as piles with periodic turning or as static piles without turning, whereas liquid manure (feces, urine, and water) was stored as a slurry in small tanks to simulate lagoon conditions. The *E. coli* and *Listeria* levels in the manure samples were determined for 29 wk. Results showed that there was an initial reduction in bacteria levels in the first month; however, both *E. coli* and *Listeria* managed to survive in the solid manure piles for the full study period. In slurry samples, *E. coli* was not detected after 14 wk, but *Listeria* survived until the end of the experiment at relatively lower levels than in the solid manure piles. Ambient weather and pile size were identified as the main reasons for bacteria survival during the course of the experiment. The outcome of this study is important in terms of understanding pathogen survival in manure piles and slurries prior to their application to crop fields.

Core Ideas

- Solid and liquid dairy manure storage conditions on pathogen decay were investigated.
- *E. coli* O157:H7 and *Listeria* spp. survived in solid manure up to 6 months.
- Pile size and temperature are factors that likely influenced pathogen survival.

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California is the leading milk-producing state in the United States with 1.74 million cows (CDFA, 2016) that produce an estimated 28.6 million Mg of manure annually. The majority of dairy facilities are located in the Central Valley of California, where flushing systems are commonly used to manage manure. During flushing, feces and urine are moved with water from housing areas, after which the slurry may flow directly to settling basins or first go through a mechanical separation system with the liquid flowing to settling basins. The liquid portion flowing from the settling basins is typically stored in earthen lagoons. The solids from mechanical separation are usually dried and, in many cases, reused as bedding. Solids collected in the settling basis may be dried, composted, or stockpiled and used as bedding or land applied as a nutrient source. Stockpiling or composting manure reduces the volume and moisture content, allowing the manure to be hauled greater distances. The lagoon liquid is applied to agricultural fields either via flood irrigation or through pressurized irrigation systems (Dungan et al., 2012). In some cases, manure is treated by more advanced systems (e.g., anaerobic digester, aerobic treatment) before being land applied (Bicudo and Goyal, 2003).

Although manure provides nutrients to crops and reduces the need for synthetic fertilizers, bacterial contamination of soil, water, and crops caused by manure application is an important issue. Dairy manure is a reservoir of pathogens such as *Escherichia coli* O157:H7, *Salmonella* spp., *Campylobacter jejuni*, *Clostridium perfringens*, and *Listeria monocytogenes*, which are a potential threat to human health (Zhao et al., 1995; Pell, 1997; Pandey et al., 2014). In manure-amended soil, pathogens can be taken up by plants through the root or transported in runoff, making their way to nearby waterbodies (Solomon et al., 2002; Tyrrel and Quinton, 2003; Oliver et al., 2005). In addition, airborne pathogens can land on food crops or surface waters (Dungan, 2010). In untreated liquid manures, pathogens may persist for longer periods of time depending on storage conditions such as low temperatures or high solids content (Diao et al., 2015). Stacking of solid manure for an extended period of time is reported to be an effective alternative for reducing or eliminating pathogens if elevated temperatures are reached (Nicholson et al., 2005).

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Abbreviations: CFU, colony-forming units.

Because much of the solid and liquid dairy manure generated is applied to crop fields, it is necessary to understand their microbial profile before using them. According to the latest surveillance study on foodborne disease outbreaks by the Centers of Disease Control and Prevention, *E. coli* O157:H7 and *Listeria monocytogenes* were responsible for nearly 25 outbreaks and >500 illnesses in 2012 (CDC, 2014). Although several studies have investigated the fate of pathogens in dairy manures (Himathongkham et al., 1999; Shepherd et al., 2010; Diao et al., 2015), few comprehensive studies have been conducted to compare the population dynamics of *E. coli* and *Listeria* in dairy manure under different storage conditions (Nicholson et al., 2005). In this context, the objective of this study was to improve knowledge on the survival of *E. coli* O157:H7 and *Listeria* spp. in dairy manure under common storage conditions, with those being turned piles, static piles, and slurry conditions.

Materials and Methods

Experimental Design, Sample Collection, and Analysis

All procedures involved in the animal trial (Niu et al., 2016) that provided manure were approved by the Institutional Animal Care and Use Committee at the University of California-Davis. The manure storage experiment was conducted in the Teaching and Research Facilities of the Department of Animal Science at University of California-Davis from August 2014 to March 2015. In the animal trial, 12 Holstein cows were randomly assigned to four dietary treatments consisting of two dietary forage levels (37.4 [low forage] vs. 53.3% [high forage] of dry matter) and 2 dietary crude protein levels (15.2 [low protein] vs. 18.5% [high protein] of dry matter). The experiment was set up as a Latin square design with four 18-d periods, with each period consisting of a 15-d adaptation period followed by a 3-d period to collect feces and urine. Feces and urine were collected separately from Period 2 through Period 4. During each collection period, feces from the dietary treatments were divided into three equivalent portions and immediately transported to the storage containers assigned to each of three manure storage treatments: turned piles, static piles, and slurry. One portion was turned once per week (turned piles), another was stacked without any additional mixing (static piles), and the remaining portion was mixed with urine from cows assigned to the corresponding dietary treatment and water at an approximate ratio of 1:3 to simulate the contents of a lagoon (slurry). The average pile diameter was 1 m and height was 0.3 m. The slurry was stored in a 379-L Poly-Tuf Tank (Freeland Industries). Manure from the three periods was treated as replicates assigned to each of 12 manure storage treatment (3) × dietary treatment (4) combinations.

The manure was stored up to 6 mo, with both ambient and manure temperature measurements taken every 15 min using a data-logging device (HOBO U23 Pro v2 External Temp Data Logger, Onset Computer Corporation). Temperature sensors were placed in the middle of the pile (for static and turned piles) or slurry tank (for slurries). Manure samples were collected from each manure storage container at Weeks 0, 1, 2, 3, 4, 9, 14, 19, 24, and 29. Sample weights from turned and static manure containers were measured at the beginning and end of their storage period. Approximately 150 g of manure was collected from the static and turned piles at random locations using a spatula. The hard surface of the static pile treatments was cut open, and four samples were collected at different locations from the bottom of the piles and then composited, after which the hard manure pieces were put back in place to reseal the piles. Water was added periodically into each slurry container once per week to maintain the initial volume of the container throughout the duration of the experiment. A 200-mL composite slurry sample was collected at various locations and depths using 50 mL Falcon polystyrene serological pipettes (Corning) immediately after stirring for 30 s. Fresh solid samples were placed in clean, sealable plastic bags, and slurry samples were placed in sterile wide-mouth sample bottles (Fisher Scientific). Samples were stored at 4°C for no longer than 24 h after collection before being analyzed for viable bacteria. The *E. coli* O157:H7 and *Listeria* spp. levels were determined using selective and differential agar media, as described by Biswas et al. (2016).

Statistical Analysis

Manure levels of *E. coli* O157:H7 and *Listeria* spp. were analyzed for manure storage effect tested at each time point in a linear mixed model with repeated measures (time) using lme4 procedure (Bates et al., 2015) of R statistical language. The model was:

$$Y_{ijklm} = \mu + M_i + \mathrm{CP}_j + F_k + T_l + P_m + M_iT_l + \mathrm{MT} + e_{ijklm}$$

where μ is the overall mean, M_i is the fixed effect of manure storage treatment (i = 1 to 3), CP_j is the random effect of dietary crude protein content (j = 1 to 2), F_k is the random effect of dietary forage content (k = 1 to 2), T_l is the fixed effect of sampling time (l = 0 to 29 wk), P_m is the random effect of manure pool (m = 1 to 3), M_iT_l is the interactions between manure storage treatment and time, MT is the covariate of manure temperature, and e_{ijklm} residual error. Bacterial levels (colony-forming units [CFU] g^{-1} or mL^{-1}) were log-transformed before performing statistical analysis, and the zero levels were taken as 1 $\log_{10}$ CFU g^{-1} before log transformation. In all analyses, the interaction term was removed from the model if statistical difference was not shown. Data points with Studentized residuals outside of ±3.5 were considered outliers and were removed from analysis. Statistical differences were declared at $P < 0.05$, and a tendency toward significance was considered at $0.05 < P < 0.10$.

Results and Discussion

The moisture contents of the slurry and manure piles are presented in Fig. 1A. After 29 wk, there was about a 60% reduction of the moisture content in the turned manure compared with a 10% reduction in the static manure. The moisture level of the slurry was maintained at ~95% throughout the experiment to maintain the initial volume and simulate the manure wastewater in a lagoon. The pH values were measured for the slurry samples over the storage period, and they varied from 7.1 to 7.8. The temperatures inside the slurry and manure containers followed a similar trend during the study (Fig. 1B). On average, the temperature of the slurry was found to be a few degrees lower than in the solid manure piles. The temperature inside the solid manure piles reached a maximum of nearly 30°C after the first 4 wk (August–September), most likely due to microbial activity, and then it decreased steadily to 15°C by Week 29. During the

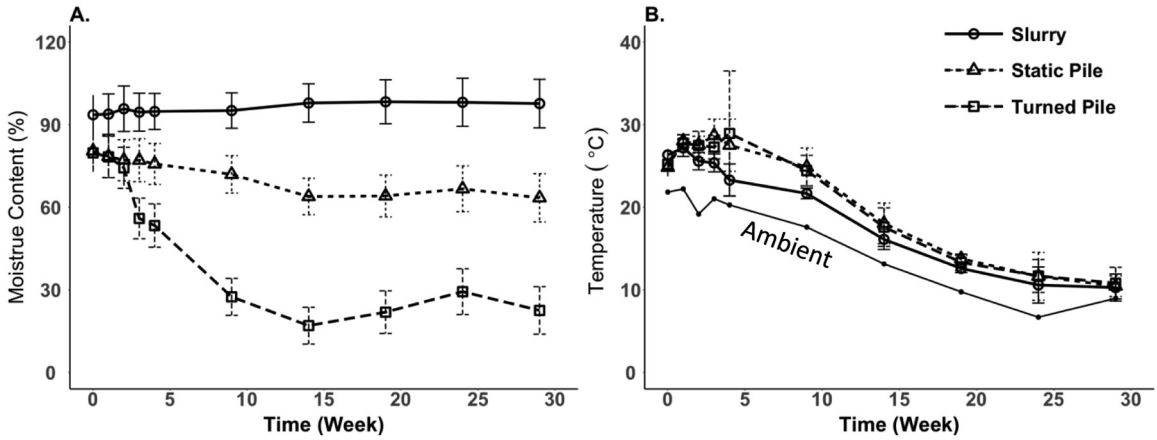

Fig. 1. (A) Moisture content (%) and (B) temperature (°C) of the manures under different storage conditions.

experiment, the initial ambient temperature was ~25°C at the start of the experiment, which gradually decreased to 10°C by the end of March.

Results from the statistical analysis found no significant impact of diets (crude protein and forage levels) on the bacteria levels during manure storage and were thus ignored in the summary table (Table 1). However, there was a significant interaction between manure storage treatment and storage time ($P < 0.01$) on the levels of *E. coli* O157:H7 and *Listeria* spp. in dairy manure (Table 1). The least square means of *E. coli* O157:H7 levels in the manure piles and slurry at different time points are presented in Table 2, along with the pairwise comparisons among the treatments. During the first 4 wk of experiment, the *E. coli* levels in the turned and static manure piles experienced the largest decrease from 7.4 to 5.5 $\log_{10}$ CFU g^{-1} and 7.2 to 3.1 $\log_{10}$ CFU g^{-1}, respectively, which coincide with an increase in temperature inside the solid manure piles. During the same time period in the slurry, *E. coli* levels decreased from 6.3 to 0.5 $\log_{10}$ CFU mL^{-1}; however, the temperature of the slurry was several degrees lower than that in the solid manure piles. After that, there was a general increase in *E. coli* levels in samples from all three storage conditions. In the turned and static piles, after Week 5, there was a sudden increase in *E. coli* levels until Week 9, but then they decreased to 4.7 and 2.7 $\log_{10}$ CFU g^{-1}, respectively, by Week 29. Similarly, the *E. coli* levels increased slightly after Week 5 in the slurry but then exhibited a steady decline to near-undetectable levels from Week 19 onward.

Table 1. Effects of manure storage conditions and time on *E. coli* O157:H7 and *Listeria* levels.

Pathogen	*P*-value†			
	M	T	MT	M × T
E. coli O157:H7	<0.01	<0.01	0.03	<0.01
Listeria	<0.01	<0.01	0.07	<0.01

† M, manure storage treatment; T, sampling time; MT, covariate of manure temperature; M × T, interactions between manure storage treatment and time.

Previous studies have reported inconsistencies in the decay pattern of *E. coli* in cattle manure in aerobic and anaerobic environments (Kudva et al., 1998; Pandey and Soupir, 2011). After inoculating bovine feces with a high concentration of *E. coli* O157:H7 (10^9 CFU g^{-1}), Kudva et al. (1998) found that the bacterium survived longer at low incubation temperatures of −20, 4, and 23°C compared with 37, 45 or 70°C under nonaerated conditions and in aerated piles. When the manure was exposed to ambient conditions, the survival of *E. coli* O157:H7 was prolonged to 47 d. While incubating farm effluents under laboratory conditions, the authors did not find any correlation between incubation temperatures or the survival of *E. coli* O157:H7 and pH. In another study, Nicholson et al. (2005) reported that the survival of *E. coli* O157 in dairy slurry from 1 to 3 mo was dependent on the dry matter content. In turned and static manure heaps, *E. coli* survived for 4 and 8 d, respectively, at ambient temperatures (<20°C). In agreement with the

Table 2. Detailed statistical analysis on the $\log_{10}$–transformed *E. coli* O157:H7 levels under different manure storage conditions over the study period.

Week	Manure treatment: LSM†			SE	*P*-value contrasts		
	T	ST	SL		T vs. SL	T vs. ST	SL vs. ST
	—— $\log_{10}$ CFU g^{-1} ——		$\log_{10}$ CFU mL^{-1}				
0	7.4	7.2	6.3	0.5	0.99	1.00	0.99
1	7.2	7.3	5.5	0.6	0.60	1.00	0.45
2	6.9	6.9	2.2	0.6	<0.01	1.00	<0.01
3	6.9	4.8	1.1	0.6	<0.01	0.21	<0.01
4	5.5	3.1	0.5	0.6	<0.01	0.21	<0.01
9	6.6	4.6	1.1	0.5	<0.01	0.21	<0.01
14	6.0	4.8	0.9	0.5	<0.01	0.98	<0.01
19	5.7	4.3	–	0.6	<0.01	0.96	<0.01
24	5.6	4.1	–	0.7	<0.01	0.86	<0.01
29	4.7	2.7	–	0.7	<0.01	0.17	<0.01

† LSM, least square mean; SE, standard error; T, turned pile; ST, static pile; SL, slurry.

Table 3. Detailed statistical analysis on the $\log_{10}$–transformed *Listeria* levels under different manure storage conditions over the study period.

Week	Manure treatment: LSM†			SE	*P*-value contrasts		
	T	ST	SL		T vs. SL	T vs. ST	SL vs. ST
	—— $\log_{10}$ CFU g^{-1} ——		$\log_{10}$ CFU mL^{-1}				
0	2.3	2.1	1.3	0.3	0.17	1.00	0.51
1	1.7	2.0	1.2	0.3	0.99	1.00	0.40
2	1.2	1.3	0.8	0.3	0.99	1.00	0.99
3	0.8	1.4	0.8	0.3	1.00	0.96	0.90
4	1.9	1.9	1.0	0.3	0.63	1.00	0.61
9	2.4	2.0	1.2	0.3	0.02	1.00	0.82
14	3.5	2.8	1.3	0.3	<0.01	0.72	<0.01
19	3.3	2.6	1.1	0.3	<0.01	0.67	<0.01
24	3.0	2.3	1.5	0.3	<0.01	0.76	0.67
29	3.2	2.6	1.1	0.3	<0.01	0.99	<0.01

† LSM, least square mean; SE, standard error; T, turned pile; ST, static pile; SL, slurry.

present study, previous studies have reported that some members of the *E. coli* O157:H7 serotype have a high tolerance to acidic or dry conditions and thus can survive longer (Wang et al., 1996). Assessing the impacts of temperature on pathogen survival, Himathongkham et al. (1999) observed a linear decay of *E. coli* O157:H7 with periodic multiplication in solid manure and slurry at different temperatures (4, 20, and 37°C). The decimal reduction time (1 log or 90%) was 1 to 3 wk in manure and 1 to 5 wk in slurry. In addition to temperature, the moisture content of the feedstock is also considered an important factor that influences bacterial growth and survival, as discovered in biosolids (Ward, 1981; Sidhu et al., 1999).

The level of *Listeria* spp. in the solid manure piles and slurry at different time points are presented in Table 3, along with the pairwise comparisons. In the case of *Listeria* spp., after the first 3 wk of the experiment, the levels decreased from 2.3 to 0.8 $\log_{10}$ CFU g^{-1} and 2.1 to 1.4 $\log_{10}$ CFU g^{-1} in the turned and static piles, respectively. In the slurry, the decay rate was slower, as it decreased from 1.3 to 0.8 $\log_{10}$ CFU mL^{-1} after 3 wk. Like with *E. coli* O157:H7, the *Listeria* levels started to increase after this period in both manure storage piles and slurry. In all three manure treatments, a steady increase in the *Listeria* level was observed from Week 3 to 14, and then by the end of experiment, they were 3.2 $\log_{10}$ CFU g^{-1} (turned), 2.6 $\log_{10}$ CFU g^{-1} (static), and 1.1 $\log_{10}$ CFU mL^{-1} (slurry). *Listeria* has been shown to survive in dairy manure for an extended period of time (Nicholson et al., 2005; Erickson et al., 2014a, 2014b). Kearney et al. (1993) found a 90% reduction of *Listeria* in dairy slurry after 29 d of storage at 17°C at a pH ranging from 7.0 to 7.6. Nicholson et al. (2005) observed that *Listeria* survival was 4 d in both turned and unturned dairy manure piles, while it was found to survive for >6 mo in dairy slurry. Erickson et al. (2014b) found different results in cow manure (compost mixture with wheat straw and cottonseed meal) that were stored at 30°C for 4 wk. The authors reported a steady decrease of *Listeria* (3.5 to 1.3 log CFU g^{-1}) during 4 wk of storage. Similarly, while incubating dairy manure with sawdust or wheat straw at 25°C, Grewal et al. (2006) found that the pile temperature did not exceed 32°C and it took 2 to 8 wk until the *E. coli* and *Listeria* were undetectable. They also observed that while storing liquid manure at room temperature (20–25°C), it took 8 wk to eliminate all viable pathogens.

Regrowth of pathogenic bacteria in dairy manures is reported widely, particularly if incomplete inactivation occurs due to low temperatures and/or incomplete organic matter stabilization caused by drying (Gibbs et al., 1997; Kim et al., 2009). When the temperature is below thermophilic range (<55–60°C), the possibility of pathogen survival is relatively high because of pile heterogeneity, manure types, source of organic materials, moisture content, and weather conditions (Shepherd et al., 2007, 2010). It is evident from the temperature profiles that the manure piles in the present study did not reach composting temperatures. The small size of the manure piles was likely crucial because it did not allow for sustained microbial activity and generation of heat necessary to kill the bacteria. In addition, periodic manure pile turning did not facilitate the required conditions for proper composting, especially since the moisture content was not maintained. In terms of manure pile stewardship, our study supports the use of composting to rapidly kill pathogens, since our turned and static piles did not eliminate *E. coli* O157:H7 and *Listeria* even after 29 wk. According to current USDA guidance, proper composting can be achieved at a minimum temperature of 55°C for 3 d in an aerated static pile. Therefore, if livestock manures are not properly treated to inactivate pathogens before land application, then there is a higher risk of contaminating crops and nearby waterbodies. The outcome of this study is important in terms of understanding pathogen survival in manure piles and slurries prior to their application to crop fields.

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