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Survival of *Enterococcus faecium* in Turkey Litter Under Different Temperature and Moisture Combinations

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ABSTRACT

Untreated poultry litter introduces a substantial load of fecal pathogens to the environment, impacting agriculture and ecosystem function. There is substantial evidence that temperature and moisture are the primary drivers of fecal bacteria survival across ecosystems. However, both temperature and moisture effects have been shown to be modulated by the matrix in which the fecal bacteria are living. This context dependence highlights the importance of understanding fecal bacteria survival in a variety of matrices in order to implement effective waste management plans. In this study, the survival patterns of *Enterococcus faecium* in post-use turkey litter under two levels of temperature (5°C, 30°C) and moisture (<10% w/v, ~35% w/v) were determined. Under both moisture conditions at 5°C, *E. faecium* abundance did not change over the course of 14 days. However, at 30°C, the low moisture treatment resulted in 23% decrease in *E. faecium* concentration over 14 days, while the high moisture treatment resulted in 16% growth over the course of the experiment. Since high temperature is usually sufficient to result in fecal bacteria decay in other matrices, this study highlights the context dependence of fecal bacteria survival. These findings support current manure management recommendations to apply litter during warm months, when soil is not wet.

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INTRODUCTION

Each year, Virginia poultry farms produce millions of kilograms of poultry waste (Pelletier et al. 2001) which contain high concentrations of fecal pathogens (Kelley et al. 1995; Martin et al. 1998; Terzich et al. 2000). Poultry litter, a combination of excreta, bedding, feathers, and feed, is a cost-effective fertilizer (Bosch and Napit 1992), which can be used to produce higher crop yields due to its excess nitrogen (N) and phosphorus (P) content (Wood et al. 1993; Evers 1998; Nyamasoka et al. 2017). However, livestock wastes have also been shown to contaminate crops (Heaton and Jones 2008; Atidégla et al. 2016; Pizarro et al. 2019) and freshwaters (Haack et al. 2016), as well as impacting ecosystem functioning (McBride et al. 2020; Wepking et al. 2017). In order to reduce the negative effects of fecal contamination and protect Virginia's natural resources and agricultural production, it is important to understand the dynamics of fecal bacteria survival in poultry litter.

In terrestrial environments, temperature and moisture have long been known to drive survival of fecal bacteria (Kibbey et al. 1978; Zibilske and Weaver 1978; Reddy et al. 1981), including pathogens (Zibilske and Weaver 1978; Cools et al. 2001). Despite the fact that high temperatures are related to increased decay of fecal bacteria (Blaustein et al. 2013), temperature was found to be the best predictor of their presence in a watershed (Badgley et al. 2019). Dry soil is generally unfavorable for the survival of fecal bacteria (Donsel et al. 1967; Pepper et al. 1993; Staley et al. 2016). It is possible, however, for these organisms to regrow once soil is moistened (Chandler and Craven 1980; Hartel et al. 2005). While it is clear that temperature and moisture drive fecal bacteria's survival in soil and water, the effect of these environmental variables in poultry litter remains unclear. Indeed, the survival of fecal bacteria can be modulated by matrix-specific characteristics. For example, diurnal temperature oscillations were shown not to affect the survival of fecal bacteria in soils with high clay content but were important in soils with high sand content (Smith et al. 2019). Likewise, saturated sand at beaches with smaller grains supported fecal bacteria for longer periods than saturated soil with larger grain size (Staley et al. 2016). These matrix-specific effects highlight the importance of understanding the survival dynamics of fecal bacteria in diverse environments.

Previous studies have investigated survival of fecal bacteria after poultry litter has been applied to land (Gu et al. 2019; Neher et al. 2019), as well as the effect of different temperature dependent treatments on removing fecal bacteria from stored poultry litter (Ngodigha and Owen, 2009; Wilkinson et al. 2011). Treatment of poultry litter is not always completely successful (Hartel et al. 2005; Neher et al. 2015), and animal manure is not always required to be treated before application as a fertilizer (USDA 2015a). Therefore, when poultry litter is applied in agricultural settings, fecal organisms enter the ecosystem in addition to the desired nutrients.

This study aims to determine how temperature and moisture affect the survival of the fecal indicator bacterium (FIB) *Enterococcus faecium* in turkey litter. Turkey litter is of particular importance to Virginia since the state is the 6th highest producer of turkeys in the United States (USDA 2020), and survival of fecal organisms does not appear to have been tested in this matrix, even though it is commonly used as fertilizer. In order to determine the effects of temperature and moisture on *E. faecium* survival we used a full factorial laboratory experiment with two levels of temperature, and two levels of moisture. We predicted that *E. faecium* decay would be facilitated

by both high temperature and low moisture and that survival would be promoted by low temperature and high moisture conditions.

METHODS

Study Site and Sample Collection

All turkey bedding and litter samples were acquired from a poultry house on a farm in Shenandoah County, VA. The litter was composed of hardwood shavings, a common material, that were reused between each flock for three successive flocks of 7,000 to 9,000 turkeys each. In between each flock the litter was windrow-composted, and each windrow was turned approximately every 72 hours for a total of three turns. Six individual composite samples were collected by combining six grab samples from the top five cm of bedding, homogenizing them, and storing at 4°C during transport to the laboratory. The gravimetric water content was determined by drying 10 g of litter from each composite sample at 105°C for 24 h.

Study Organism Description

Enterococci were isolated from individual composite samples using EPA Method 1600 (US EPA, 2014) and modified for use with poultry litter. Briefly, the contents of the microcosm were added to 500 mL of PBS in a 1 L shaker flask, and bacteria were dispersed by shaking for one hour using a Burrell Wrist Action Shaker™ (75-775-24; Model 75, Pittsburgh, PA) (Zuberer 1994), then allowed to sit for 30 minutes to allow debris to settle out of solution. While it is likely that some of the suspended bacteria also settled during this time it should have done so at a similar rate between treatments. Supernatant was serially diluted and filtered onto a 0.45 µm nitrocellulose filter, which was placed on membrane-Enterococcus Indoxyl-β-D-Glucoside (mEI) Agar (215047; Becton, Dickinson and Company, Sparks, MD) and allowed to incubate 24 h at 41°C, before being counted. Putative enterococci presented a blue hue. A subset of blue isolates was selected from plates and identified to species using the Biolog System, following the manufacturer's instructions (71102; Biolog, Inc., Hayward, CA).

Enterococcus faecium was identified as the most common species isolated from these samples (data not reported), and American Type Culture Collection 19434 was used in all of the laboratory experiments. *Enterococcus faecium* is a fecal indicator bacterium (FIB) and is recommended as a proxy for pathogens by the United States Environmental Protection Agency for detecting contaminated water ways (US EPA 2012, 1986, 1976). Enterococci are Gram-positive, catalase-negative, facultative anaerobic cocci, which can grow at temperatures between 10 and 45°C, in broth containing 6.5% NaCl, and at pH 9.6. They are also able to hydrolyze esculin with the presence of bile salts, and they present the Lancefield group D antigen (Facklam et al. 2002). The wide range of environmental tolerances and their commensal presence in the poultry gut microbiota make the enterococci an ideal model system for studying the survival of fecal bacteria in poultry litter.

Microcosm Experiments

A 2 x 2 factorial experiment was conducted with two levels of temperature (5°C and 30°C) and two levels of moisture (<10% and 35% w/v water); hereto the treatments will be referred to as

'5°C/Dry', '5°C/Wet', '30°C/Dry', and '30°C/Wet'. The two temperatures were used to approximate mean temperatures in Shenandoah County, VA during the summer and winter, while the two moistures were chosen based on accounts that stored litter often has a dry outside layer while deeper in the pile there is higher moisture (Hartel et al. 2000). The experiment was performed twice.

Litter was homogenized after passing through a sieve (2 cm) and sterilized in an autoclave at 121°C for 15 minutes. We used sterilized litter to eliminate ecological interaction, such as predation, and competition with other organisms. Sterile litter was analyzed for nutrient content by The Pennsylvania State University Agricultural Analytical Service Laboratory using the methods listed in Table 1: total N using combustion analysis (Watson et al. 2003); NH_4-N using a specific ion electrode (Peters et al. 2003); P_2O_5 and K_2O using Microwave-assisted acid digestion with inductively coupled plasma spectroscopy (Wolf et al. 2003); Organic N by subtracting NH_4-N from total N.

TABLE 1: Microbiological and nutrient content of litter: including total enterococci (CFU g^{-1} dry litter); total nitrogen (N), Ammonium (NH_4-N), Organic N, phosphorus pentoxide (P_2O_5), and potassium oxide (K_2O); g kg^{-1}.

Enterococcus	**Total N**	**NH_4-N**	**Organic N**	**P_2O_5**	**K_2O**
1.39×10^5	47.3	2.9	44.4	42.0	37.5

Individual microcosms were prepared by adding ~10 g of sterile dry weight equivalent turkey litter to a 50 mL conical tube, adjusting to the appropriate moisture content, and inoculating with 1 mL of *E. faecium* inoculum (approximately 1.5×10^8 CFU mL^{-1} washed and suspended in PBS). At each time point (+1 hour, 1 day, 7 days, and 14 days) three replicate samples from each treatment were extracted in PBS following the above reported modified EPA Method 1600. For each treatment, controls were prepared by constructing five additional microcosms without the addition of inoculum, and one of these controls was harvested at each time point to verify sterility of the experiments. Throughout the experiment the uninoculated controls were below the colony counting limit. Colony forming units (CFU) were identified as blue colonies on each plate when viewed under magnification. The total CFU g^{-1} dry litter was calculated as the counted blue colonies per the plated volume multiplied by the dilution factor divided by the mass of dry litter. The litter-PBS mixture produced a significant number of suspended particles which made filtering difficult without first performing a 10^{-2} dilution; therefore, the lower counting threshold was 1.2 CFU g^{-1} dry litter.

Statistical Analyses

The total colony forming units (CFU) from the duplicate experiments were $\log_{10}$ transformed and analyzed together with a linear mixed effect model using the lme4 package (Bates et al. 2015) in R version 3.6.1 (R Core Development Team 2019). Some individual replicates were contaminated and were removed from analysis. At each time point, each treatment is represented

by four to six replicates. Temperature, moisture, and incubation time were main effects, and experiment ordinality was a random effect. The model was evaluated for normality of residuals using the shapiro.test command and the residuals were not normally distributed. To account for this, we then performed a general linear mixed effect model fitting the model to a gamma distribution and using the log-link function. We evaluated interactions between moisture and temperature by performing pairwise comparisons within each time point using the TukeyHSD method. We then evaluated the effect of incubation time by performing within treatment pairwise comparisons between the starting concentration of *E. faecium* and the measured concentration at each time point using Dunnett's test, in the *emmeans* package (Lenth et al. 2020); $\alpha = 0.05$.

RESULTS

Microcosm experiments showed a significant main effect of moisture ($P < 0.001$), temperature ($P = 0.002$), and incubation time ($P = 0.027$). There was also a significant 2-way interaction between moisture and temperature ($P < 0.001$) and moisture and incubation time ($P < 0.001$), but not between temperature and incubation time ($P = 0.76$). Finally, there was a significant three-way interaction between all main effects ($P < 0.001$).

When comparing treatments within time points, there was no significant pairwise differences ($P > 0.05$) between the starting concentrations of enterococci, which ranged from $\log_{10}$ 7.1 to 7.6 CFU g^{-1} dry litter. By Day 1, the concentration of *E. faecium* in the 30°C/ Dry treatment declined below the other treatments by 9-13% (all $P < 0.05$). On Day 7, 30°C/Dry treatment continued to contain the lowest concentration of *E. faecium*, which reached 6.1 $\log_{10}$ (CFU g^{-1} dry litter) -- 17-28% less than the other treatments (all $P < 0.001$). While decay continued to occur in the 30°C/Dry treatment on Day 7, the abrupt increase in the percent difference was largely driven by a concomitant increase in *E. faecium* concentration in the 30°C/Wet treatment. At the end of the experiment on Day 14, the 30°C/Dry treatment continued to decline reaching 5.5 $\log_{10}$ (CFU g^{-1} dry litter), 24-38% (all $P < 0.001$), and the 30°C/Wet was 17-19% greater than the two 5°C treatments (both $P < 0.001$).

We also evaluated the difference in enterococci concentration within each treatment over time to determine if there were significant changes from the starting concentration within that treatment. At 5°C, *E. faecium* levels did not change between any of the four sampling days in either the low moisture or high moisture treatments. However, at 30°C both the low moisture and high moisture treatments enterococci concentrations did change with time. At 30°C/Wet, *E. faecium* concentrations increased by 16% by the end of the experiment ($P < 0.001$), a 1.2 $\log_{10}$ CFU g^{1} dry litter increase. In the low moisture experiment, *E. faecium* concentration decreased 14% from Day 0 to Day 7 ($P = 0.007$), and 23% between Day 0 and Day 14 ($P < 0.001$), a ~1.6 log10 CFU g^{1} dry litter decrease over the course of the experiment.

DISCUSSION

Both temperature and moisture affect the survival of *E. faecium* in turkey litter. Specifically, at 30°C, *E. faecium* grow under wet conditions and decay under dry conditions (Figure 1). While we predicted that *E. faecium* would decay most rapidly in warm and dry conditions we did not necessarily expect growth under warm and moist conditions. Since other

studies have found that temperature alone can cause microbial decay (Cools et al. 2001), this study highlights the importance of understanding population dynamics of fecal bacteria in different matrices. Poultry litter may favor growth more than soil alone due to increased availability of carbon and nitrogen for building microbial biomass (Acosta-Martínez and Harmel 2006).

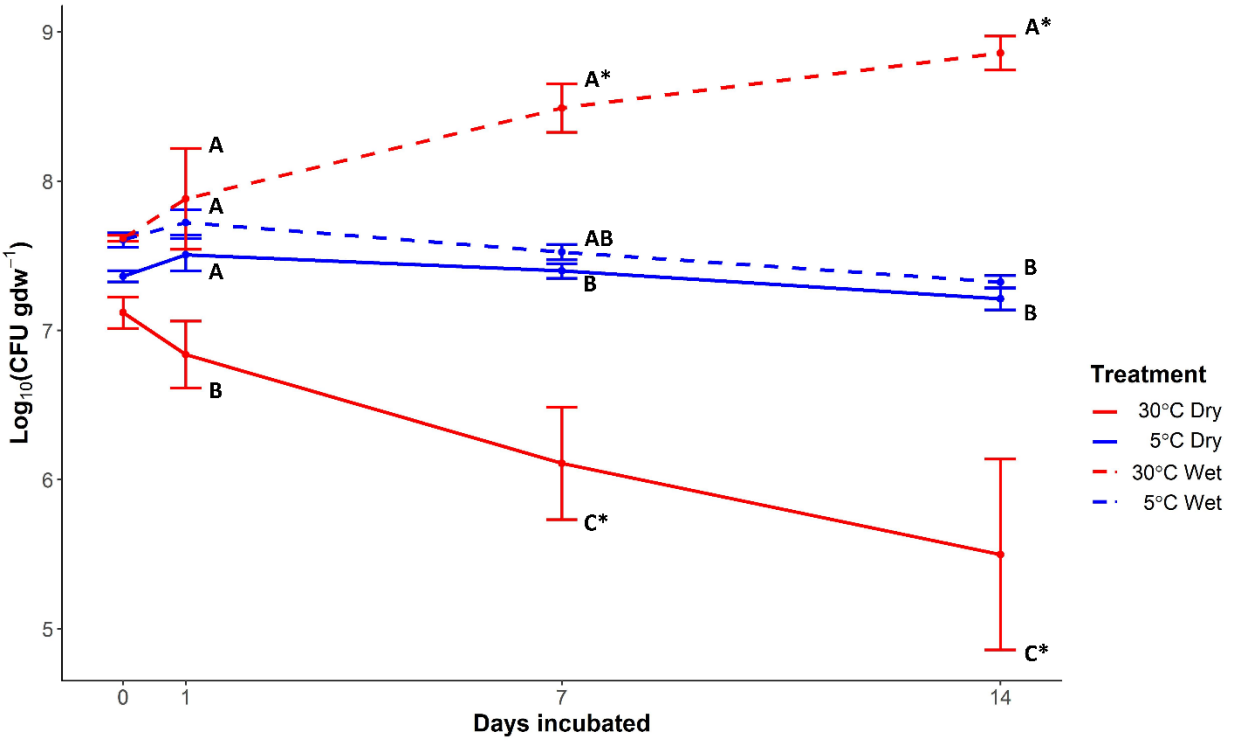

Figure 1: Time series illustrating the survival of *Enterococcus faecium* in turkey litter under different moisture and temperature combinations. Significant treatment differences within an individual time point are denoted by different letters (α=0.10). Significant differences from the starting concentration within a treatment are denoted by an asterisk (α=0.10). Shown are means ± 1 standard error for each time point.

Fecal bacteria in poultry litter have been shown to decay more rapidly when mixed with soil, but to persist when litter was not tilled into the soil (Hruby et al. 2016). It is unclear whether this is due to soil biological or edaphic properties. Due to the increased call for conservation agriculture, such as no till practices (Kassam et al. 2019), it is clear that reducing pathogen load before applying poultry manure as fertilizer is important for ecosystem function. This can be achieved through a variety of composting methods such as windrowing or deep stacking (Ngodigha and Owen 2009; Wilkinson et al. 2011). However, it can be difficult to achieve lethal temperatures throughout the entire compost pile (Tiquia and Tam 2002; Neher et al. 2015), and pathogen regrowth can sometimes occur when environmental conditions improve (Sinton et al. 2007). Additionally, since the demand for fertilizers such as poultry litter decreases during the winter, it is often stored under cooler temperatures, which could lead to longer persistence, as the

concentration of *E. faecium* did not change throughout the duration of this experiment, in either wet or dry conditions at 5°C (Figure 1). Although, this study did not mimic freeze-thaw cycling, which can increase decay of fecal bacteria (Dilek Sanin et al. 1994), the results suggest that there is negligible change in *E. faecium* at low temperature regardless of moisture (Figure 1).

This study provides information that poultry farm managers and regulators can use to determine best practices for land application of poultry litter in order to minimize bacterial impacts on the ecosystem. Future studies should continue to explore environmental and ecological factors that influence survival of enterococci in other environments that mimic field conditions as has been performed in other ecosystems, e.g., use of refugia (Badgley et al. 2010a, 2010b), or competition and predation (Wanjugi and Harwood 2013). Current USDA recommendations (USDA 2015b) for poultry litter application do not explicitly consider survival of pathogens; however, this study supports current recommendations. Poultry litter should not be applied to fields that are wet, or before a precipitation event to reduce the chances of increased moisture leading to a bloom of fecal bacteria. In addition, poultry litter should not be applied under extended cool periods since this could lead to persistence of fecal bacteria in the litter material.

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