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Bacterial Content in Runoff from Simulated Rainfall Applied to Plots Amended with Poultry Litter

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Abstract: To evaluate potential bacterial runoff from poultry litter, litter was applied to test plots and exposed to simulated rainfall 1, 8 or 15 d after litter application. Runoff samples were tested for *Salmonella* and *Campylobacter*, two bacterial pathogens commonly associated with poultry, as well as common fecal indicators such as coliforms, enterococci and *Escherichia coli*. The runoff samples were evaluated from treatments of no litter (control), or the equivalent of 1, 2 or 4 ton/acre of untreated poultry litter. Additionally, runoff samples from treatments of 2 tons/acre of alum-treated litter, 2 tons/acre composted litter and 2 ton/acre deep-stacked litter were compared for bacterial content. Three replicates of the treatments were performed, for a total of 21 test plots. No *Campylobacter* was isolated from any of the samples and the majority of samples tested negative for *Salmonella*. Although Salmonella was detected in runoff from many of the plots, it may have originated from sources other than the applied litter (rodents, birds, etc.) since it was detected in two of the unfertilized control plots.

Key words: *Campylobacter*, *E. coli*, poultry litter, runoff, *Salmonella*

INTRODUCTION

As livestock can serve as reservoirs for many zoonotic pathogens that adversely affect human health, such as *Campylobacter*, *Salmonella* and pathogenic *Escherichia coli* (Shepherd *et al*., 2009; Volkova *et al*., 2009), water contamination through the indirect introduction of animal feces is a concern. As animal waste is commonly applied to agricultural fields to improve nutrient and soil qualities (Moore and Edwards, 2007), runoff from treated fields has the potential to introduce microbes to drainage waterways, like streams and rivers (USEPA, 2009). If pathogens are introduced to watersheds, it is possible that the presence of pathogens could result in contamination of recreational and municipal drinking water (Haley *et al*., 2009) and cause human disease. Because there are so many enteric pathogens, often with specific biochemical requirements for culturing, it is common to instead measure the counts of indicator organisms (e.g., coliforms, enterococci, *E. coli*) as an indicator of watershed contaminations (USEPA, 2012).

Two of the most significant human pathogens associated with poultry are *Campylobacter* and *Salmonella*. Both *Campylobacter* and *Salmonella* colonize the gastrointestinal tract of birds (Dunkley *et al*., 2009; Horrocks *et al*., 2009) and are shed in feces (Marin and Lainez, 2009), thereby potentially contaminating the litter. *Escherichia coli* O157 is a pathogenic strain of *E. coli* not found in poultry but which may colonize cattle and swine (Hutchison *et al*., 2005). Since cattle are often associated with fields amended with poultry litter, this bacterium was also evaluated.

The objective of this study was to evaluate bacterial content in runoff water after litter application to agricultural plots. While many runoff studies employ filtration techniques (Jenkins *et al*., 2007, 2009), because of the high suspended solids in the runoff samples clog the filters, it was necessary to employ centrifugation instead to concentrate and detect bacterial counts. The litter density used in this study is typical of litter application in Northwest Arkansas and ranges between 1 to 2 tons/acre as determined by the Eucha/Spavinaw phosphorus index (DeLaune *et al*., 2006). Common litter management techniques were also evaluated and included the application of alum, composting of litter and deep-stacking litter. Alum is commonly applied to litter in poultry houses to reduce ammonia volatilization and soluble phosphorus (Moore and Edwards, 2007). Composting and deep-stacking increase ammonia volatilization and these practices are often done to decrease odors and reduce pathogen concentrations.

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MATERIALS AND METHODS

Plot characteristics: The study was conducted on agricultural test plots in Fayetteville, Arkansas; a total of 21 plots were used, each 100 sq. ft with a 5% slope on Captina silt loam soil (fine-silty, siliceous, mesic, Typic Fragiudults) and hydrologically isolated with a 15-cm metal strip. Water was collected in aluminum troughs on the down-slope edge of each plot. A minimum of 1 year had passed since any previous treatment of the test plots.

Treatments: Four treatments were applied to the 12 test plots (n = 3 plots/treatment): no litter (control), 1, 2 or 4 tons untreated poultry litter/acre. Additionally, a 2 tons/acre untreated litter application was compared with three supplementary treatments which were applied to 9 test plots (n = 3 plots/treatment): a 2 tons/acre alum-treated litter treatment, a 2 tons/acre composted litter treatment and a 2 tons/acre deep-stacked litter treatment. Three plots were used to test each treatment and were considered replicates for analysis. The litter used in the study was collected from local poultry farms in Northwest Arkansas; untreated litter was taken directly from the house and applied to the test plots the same day. Alum was applied to the alum-treated litter with the addition of each flock to the house. Composted litter was stacked, brought to 50% moisture and turned weekly. Deep stacked litter was stored in a stacking shed, undisturbed, until application to the test plots.

Litter testing: Prior to field application the litter to be applied was tested for *Campylobacter*, *Salmonella* and *E. coli* O157. Three 75 g samples were taken from each type of litter being applied: normal untreated, alum-treated, composted and deep-stacked litter. Each 75g sample was then divided into three 25 g subsamples and each subsample tested for one of the pathogens of interest.

For *Campylobacter*, a 25 g subsample was placed in 225 mL of *Campylobacter* Enrichment Broth (CEB) and then 5 mL aliquots were placed in 5 empty tubes. Serial dilutions in CEB were then performed and tubes were incubated at 37°C for 24 h followed by plating 100 μL on Campy-Line Agar (CLA) and incubating at 42°C for 48 h.

For *Salmonella*, a 25 g subsample was placed in 225 mL of Buffered Peptone Water (BPW) and incubated at 37°C for 24 h and then 0.5 mL aliquots were placed in 5 tubes containing 4.5 mL of tetrathionate (Hajna) broth (TT broth). Serial dilutions in TT broth were then performed and tubes were then incubated at 37°C for 24 h. Ten microliters were then streaked on both Brilliant Green Sulfa agar (BGS; Becton, Dickinson and Company, Sparks, MD, USA) and Modified Lysine Iron Agar (MLIA; Becton, Dickinson and Company, Sparks, MD, USA) and incubated at 37°C for 24 h.

For *E. coli* O157, a 25 g subsample was placed in 225 mL of Lauryl Tryptose Broth (LTB; Becton, Dickinson and Company, Sparks, MD, USA) and then 5 mL aliquots were placed in 5 empty tubes. Serial dilutions in LTB were then performed and tubes were incubated at 37°C for 24 h. Ten microliters were then streaked on Sorbitol MacConkey agar (SMAC; Becton, Dickinson and Company, Sparks, MD, USA) and incubated at 37°C for 24 h. From the SMAC 1 to 3 suspected colonies were transferred to Brain Heart Infusion agar (BHI), which was incubated at 37°C for 24 h and a single colony was transferred to a 5 mL tube of LTB-MUG. The tube of LTB-MUG was incubated at 37°C for 24 h and then observed for gas production and negative fluorescence. Positive tubes were considered potential positives.

Suspected colonies of *Campylobacter*, *Salmonella*, or *E. coli* O157 were identified using the Biolog System (Biolog Inc., Hayward, CA, USA), API (bioMerieux, Hazelwood, MO) and latex-agglutination with CAMPY (jcl)™ *C. jejuni* Latex Agglutination Assay (Scimedx Corporation, Denville, NJ, USA), Difco™ BBL™ *Salmonella* O Antiserum Group Poly A-I and Vi (Becton, Dickinson and Company, Sparks, MD, USA) and Dry Spot *E. coli* O157 test kits (Oxoid, Cambridge, UK).

Sample collection: Simulated rainfall was applied to test plots at the rate of 5 cm/h until the first runoff was observed from each plot (average 37 min) and continued for 30 min thereafter. Rainfall was simulated on each plot weekly for 3 weeks (D1, D8 and D15), beginning the day after litter application (D1). Rainfall was simulated using a large rainfall simulator with eight TeeJet 1/2HH-SS30WSQ nozzles (Spraying Systems Co., Wheaton, IL) approximately 3 m above the soil.

Indicator organisms: Runoff samples were tested for coliforms, enterococci and *E. coli* using Quanti-Tray®/2000 Colilert® and Enterolert® kits (IDEXX Laboratories, Westbrook, ME, USA).

Pathogens: To concentrate bacterial pathogens, 2.4 L (maximum capacity of the laboratory's centrifuge) of runoff per plot was centrifuged for 10 min at 2000 g and the supernatant extracted. The pellet was then reconstituted with 5 mL of phosphate buffered saline (PBS; pH 7.4), serially diluted in 5 ten-fold dilutions of PBS and 100 μL of each dilution plated on CLA for *Campylobacter*, BGS and MLIA for *Salmonella* and Rainbow® O157 agar (Biolog Inc., Hayward, CA, USA) for *E. coli* O157. CLA was incubated at 42°C for 48 h; BGS, MLIA and Rainbow plates were incubated at 37°C for 24 h. Bacterial counts were recorded and converted to CFU/100 mL for clarity of presentation. Suspected colonies of *Campylobacter*, *Salmonella*, or *E. coli* O157 were identified as described above. One mL of each sample was inoculated into 9 mL of tetrathionate

(Hajna) broth as an enrichment for *Salmonella*, in addition to plating. The enrichment was incubated at 37°C for 24 h and then plated on BGS and MLIA and incubated for an additional 24 h.

Statistical analysis: Data were analyzed by ANOVA using the GLM procedure of SAS (SAS Institute, 2002). The number of pathogen colonies counted were logarithmically transformed (log_{10} CFU/100 mL) before analysis to achieve homogeneity of variance (Byrd *et al*., 2001). Treatment means were partitioned by LSMEANS analysis (SAS Institute, 2002). A probability of $p<0.05$ was required for statistical significance.

RESULTS

Litter prescreening: Prior to field application, no litter sample tested positive for *Campylobacter*, *Salmonella*, or *E. coli* O157:H7.

Indicator organism, coliforms: There were no differences between coliform counts for the controls or any of the treatment groups. The mean coliform counts of all plots were 7.2-log at D1, 6.5-log at D8 and 5.8-log at D15.

Indicator organism, enterococci: Enterococci counts were significantly higher in the 4 tons/acre treatment when compared to the no litter control treatment at D1, but not at D8 or D15 as shown in Fig. 1. There were no other differences in enterococci counts in runoff samples when compared with the untreated control plots

Litter treatment: There was no significant difference in enterococci counts in runoff samples from the 2 tons/acre untreated litter, 2 tons/acre alum-treated litter, 2 tons/acre composted litter and 2 tons/acre deep-stacked litter treatments.

Indicator organism: *Escherichia coli*: *E. coli* counts were significantly higher in the 1 tons/acre treatment when compared to the no litter control treatment at D1, but not at D8 or D15 as shown in Fig. 2. There were no other differences in *E. coli* counts in runoff samples when compared with the untreated control plots.

Litter treatment: There was no significant difference in *E. coli* counts in runoff samples from the 2 tons/acre normal litter, 2 tons/acre alum-treated litter, 2 tons/acre composted litter and 2 tons/acre deep-stacked litter treatments.

***Campylobacter, E. coli* O157:H7 or *Salmonella*:** No *Campylobacter* or *E. coli* O157:H7 was isolated from any runoff sample. *Salmonella* was isolated from 14 runoff samples, with an average of 2 positive samples per treatment. *Salmonella* was isolated from runoff samples from every treatment group, including the litter

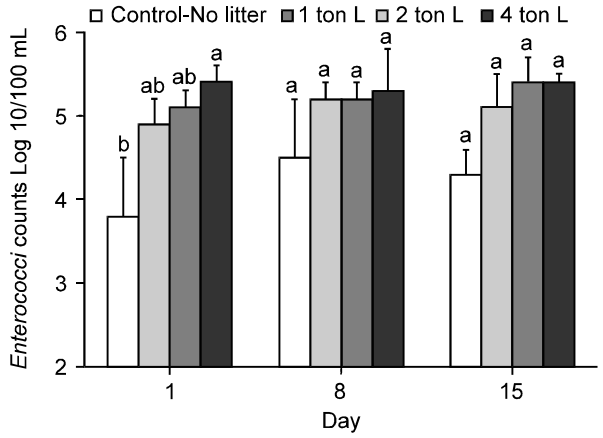

Fig. 1: *Enterococci* counts/100 mL of runoff water from poultry litter density treatments (Mean±SEM). Treatments with different superscripts within days differ significantly ($p<0.05$)

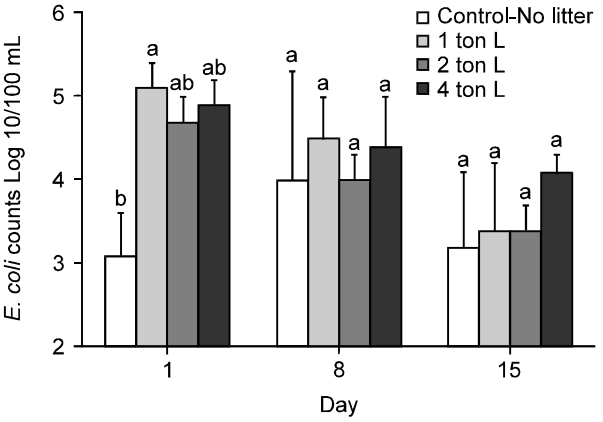

Fig. 2: *E. coli* counts/100 mL of runoff water from poultry litter density treatments (Mean±SEM). Treatments with different superscripts within days differ significantly ($p<0.05$)

Table 1: *Salmonella* counts in water runoff samples from plots with different densities of poultry litter

	Control (no litter)	1 ton L	2 ton L	4 ton L
Plot 1				
Day 1	2.7	*	*	*
Day 8	*	*	*	*
Day 15	*	*	*	*
Plot 2				
Day 1	*	*	*	*
Day 8	*	*	*	2.7
Day 15	DE	*	DE	3.0
Plot 3				
Day 1	*	*	*	*
Day 8	4.9	5.2	*	4.9
Day 15	*	4.8	*	*

DE = detectable with enrichment media

* = Non detectable

Table 2: *Salmonella* counts in water runoff samples from plots with 2 tons of poultry litter subjected to different treatments

	2 tons			
	Untreated L	L with Alum	Composted L	Deep stack L
Plot 1				
Day 1	*	*	*	DE
Day 8	*	*	*	*
Day 15	*	*	*	3.4
Plot 2				
Day 1	*	*	*	*
Day 8	*	*	*	*
Day 15	DE	*	2.5	*
Plot 3				
Day 1	*	*	*	*
Day 8	4.9	5.2	*	4.9
Day 15	*	4.8	*	*

DE = detectable with enrichment media
* = Non detectable

negative control, as shown in Table 1 and 2, but not from every plot (replicate) or every sample from a litter treated plots.

Salmonella was isolated from two samples at D1, five samples at D8 and seven samples at D15 for both the various amounts of litter (Table 1) or litter treatments (Table 2). Interestingly, the litter-negative control group yielded two positive samples, the average number of positive samples among all treatment groups. For the 14 positive samples collected, 4 were non-detectable with direct plating and were detected only with the enrichment step and thus impossible to accurately enumerate.

DISCUSSION

In this study, coliform counts were not indicative of the density of poultry litter in field runoff, despite the expectation that higher densities of litter application might result in higher counts of coliforms. Coliform counts are frequently used as a measure of water quality and have been used as a tool in determining the safety of water (USEPA, 2009, 2012). Recent research, however, has shown that coliforms are of dubious value as an indicator of water-safety (Brooks *et al*., 2009; USEPA, 2012).

When enterococci and *E coli* counts were evaluated in runoff water samples, only the 1 or 4 ton litter application were different than the litter free control plots, respectively (Fig. 1 or 2). Moreover, this difference was only detected on the first day and not in runoff water from the eight or fifteen day after application (Fig. 1 or 2). Enterococci are commonly used as an indicator of water contamination (USEPA, 2012) and have been reported to be more accurate than coliforms (Brooks *et al*., 2009). It appears that, in most cases, litter application had a limited effect on enterococci and *E coli* counts in runoff water and may not be a good indicator of water-safety (Jenkins *et al*., 2011, 2012).

***Campylobacter* and *E. coli* O157:** Neither *Campylobacter* nor *E. coli* O157:H7 were isolated from any sample, litter or runoff, throughout the experiment. *E. coli* O157:H7 is normally associated with cattle and therefore its absence in both poultry litter and runoff samples is not unexpected. *Campylobacter*, however, is known to frequently colonize the gastrointestinal tract of poultry and likewise was not found. The absence of *Campylobacter* in litter samples is probably due to its fragility outside the gastrointestinal tract (Park, 2002).

***Salmonella*:** Similar to *Campylobacter* and *E. coli* O157:H7, *Salmonella* was not isolated from poultry litter prior to the placement on the plots. When litter was applied to plots and rained on, *Salmonella* was detectable in 2/9, 1/9, 2/9 or 3/9 possible occasions for the control, 1, 2 or 4 ton litter applications, respectively (Table 1). *Salmonella* was detectable on only 2/9, 2/9, 1/9, or 3/9 occasions for the untreated, alum, composted or deep stacked litter, respectively (Table 2). These results suggest there are no clear differences for *Salmonella* runoff between untreated plots or plots receiving various amounts (Table 1) or treatments (Table 2) of poultry litter. With regards to the origin of the *Salmonella* in the runoff, since it was not possible to test all the litter for *Salmonella* prior to placement, it was possible some litter was contaminated and contributed to runoff. However, since the untreated plots also had comparable *Salmonella* runoff prevalence to litter treated plots, it is probable that the *Salmonella* was from other environmental sources. Many animals (e.g., cattle, deer, raccoons, etc) and some insects (e.g., beetles) can shed *Salmonella* into the environment which may be the cause of the contaminated runoff water (de Freitas *et al*., 2003; Renter *et al*., 2006; Compton *et al*., 2008; Roche *et al*., 2009).

Conclusion: Poultry litter application rates, litter treatments and the number of days after rainwater application appears to have a limited, if any, effect on enterococci, *E coli* or *Salmonella* counts in runoff water when compared with water collected from control plot without litter. *Campylobacter* and *E. coli* O157:H7 were not detectable in any samples. It is interesting that the human pathogen, *Salmonella*, was found in rainwater samples from litter free control plots. It is probable that the source of this *Salmonella* is environmental. In field and watershed studies wildlife is often implicated as a significant source of microbial and pathogenic contamination (Haley *et al*., 2009; Jokinen *et al*., 2010).

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