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Occurrence, antibiotic resistance, and survival of fecal enterococci in turkey litter

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Occurrence, Antibiotic Resistance, and Survival of Fecal Enterococci in Turkey Litter

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Dedication

To my loving wife, Jennifer McBride, who has supported me financially, and emotionally throughout our relationship and during my graduate work. To my daughter, Arianna Echo McBride, who has inspired me to be someone she would be proud to call her father. To my parents, Steven Glynn McBride I, Mary Franklin Cupp, Wayne Cupp, John Paul Jones, and Harriett Jones, my brothers Christopher Michael Jones, Michael James McBride, and Kody Mykl McBride, and my grandparents Quilla Dean Reidell, Edward Reidell, Peggy Ann Kimrey and Clarence Addison Kimrey for supporting me throughout my higher education.

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Abstract

The United States Environmental Protection Agency's National Water Quality Inventory and the Commonwealth of Virginia's 305(b)/303(d) Water Quality Assessment Integrated Report show fecal bacteria to be the most common cause of impairment for both streams and estuaries. Human and animal sources have both been identified as significant contributors of pathogenic bacteria to surface waters. In this study, turkey litter from a farm in Shenandoah County, VA was surveyed for total culturable bacteria and total culturable enterococci before and after a transition to organic rearing practices. The enterococci were identified to species phenotypically using the Biolog Microbial Identification System and resistance to twelve antibiotics (ampicillin, doxycycline, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, linezolid, quinipristin/dalfopristin, rifampin, streptomycin, tetracycline, vancomycin) was determined using the Kirby-Bauer disc diffusion method with automated image analysis using a Biomic™ plate reader. The effect of temperature (5°C, 30°C) and moisture (<10% H_2O, ~35% H_2O) on the survival of *Enterococcus faecium* in turkey litter was determined by inoculating sterilized turkey litter with a stock culture of bacteria and quantifying colony forming units over time. The transition to organic rearing practices resulted in a reduction in the proportion of enterococci resistant to doxycycline, gentamicin, and tetracycline, and an increase in the proportion of enterococci resistant to ciprofloxacin, erythromycin, and streptomycin. Enterococci isolated from the litter of organically raised birds were resistant to fewer antibiotics than enterococci isolated from the litter of conventionally raised birds. There was an interaction between moisture and temperature on the survival of *E. faecium* in turkey litter, with bacteria levels dropping most quickly in warm and dry conditions. The transition to organic practices has an immediate effect on antibiotic resistance patterns in enterococci, including an overall reduction from 4.02 to 3.45 antibiotic resistance phenotypes. Results of this study indicate that when applying turkey litter to land, fecal bacteria will likely survive for prolonged periods especially in cool moist conditions; therefore, it is

recommended that prior to land application bacteria loads in litter should be reduced by composting or other treatment.

Introduction

The United States Environmental Protection Agency's (US EPA) National Water Quality Inventory report and the Commonwealth of Virginia's 305(b)/303(d) Water Quality Assessment Integrated Report show fecal bacteria to be a leading cause of impairment for both streams and estuaries (US EPA 2009a; VA DEQ 2014). Human and animal sources have both been implicated as significant contributors of pathogenic bacteria to surface waters (Ferguson 2003; Tallon 2005). When recreational water bodies and drinking water sources are contaminated with fecal bacteria they pose a serious public health risk (US EPA 2009b, 2009c). Outbreaks of gastroenteritis and other illnesses have occurred due to fecal contamination of marine, fresh, recreational, and groundwater (Baron 1982; Berg 2000; Fleisher 1996; Koh 2011). These outbreaks can be attributed to the high density of pathogenic bacteria found in feces (Zoetendal 2004). A disparate group of pathogens may be present, including the bacteria *Escherichia coli*, *Salmonella* spp., *Staphylococcus* spp., *Listeria* spp., *Campylobacte*r spp., *Clostridium* spp.; the protozoa *Cryptosporidium parvum,* and *Giardia* spp.; and the viruses enterovirus, and reovirus (Cox 2005; Hutchison 2005a; Ngodigha 2009).

Animal manure has been shown to frequently be a source of fecal contamination of surface water (Heaney 2015; Mallin 2015). One type of manure of particular interest in the Shenandoah Valley of Virginia is poultry litter, a combination of bedding, excreta, feathers, and feed. A diverse array of bedding materials has been evaluated for their effects on poultry growth and health (Atapattu 2007; Atencio 2010; Benabdeljelil 1996; Davis 2010; Sarica 2000; Swain 2000; Villagrá 2011; Willis 1997). However, the

microbiology of novel litter materials is often ignored during evaluation, other than investigating pathogens that directly affect the health of the birds.

Bacterial levels in poultry litter generally rise steadily during the growth of the birds until finally leveling off after the first few weeks (Halbrook 1951; Macklin 2005). Bacteria excreted in feces are likely able to multiply in the litter environment (Schefferle 1965). In one of the few examples where different poultry litter types were directly compared based on their microbiological quality, sand was found to harbor slightly lower levels of aerobic, anaerobic, and enteric bacteria than wood shavings (Macklin 2005). In contrast, another study found no microbiological differences between a variety of poultry litter materials (Schefferle 1965).

Approximately 90% of the used poultry litter produced in the United States is applied to land (Mitchell 2005; Moore 1995; Sharpley 1993). This serves the dual purpose of disposing of large quantities of waste while simultaneously enriching the soil, as the litter contains high concentrations of available nitrogen and phosphorous, making it an effective fertilizer (Preusch 2002). Fertilizers from livestock waste can improve overall crop yields and are often cheaper than their industrial counterparts (Haynes 1998). However, in addition to adding nutrients to the soil, used litter carries high levels of aerobic and anaerobic bacteria (Halbrook 1951; Lovett 1971; Macklin 2005; Martin 1998; Nodar 1990; Schefferle 1965; Soupir 2006; Terzich 2000). Enterococci and pathogenic bacteria are prevalent in poultry litter, with enterococci levels reaching as high as 10^6 CFU/g of litter at the end of rearing cycles (Brooks 2010; Schefferle 1965). Several species of enterococci have been isolated from the gut and litter of poultry with *E. faecium* and *E. faecalis* most commonly being the dominant species (Debnam 2005;

Devriese 1991; Graham 2009; Sapkota 2011). These fecal borne bacteria, if carried in used litter and spread over land, can ultimately contaminate surface and drinking water sources.

The diversity of pathogens in feces has resulted in the development of assays that detect fecal indicator bacteria (FIB) (*E. coli*, *Enterococcus* spp. and fecal coliforms) as indicators of contamination, rather than individual bacterial or viral pathogens (Noble 2003; Scott 2002; US EPA 1976). FIB are not usually pathogenic; however, they possess attributes that make them effective proxies for pathogen detection and indicators of fecal contamination (US EPA 1986, US EPA 2012). Elevated levels of FIB in recreational waters have shown a strong correlation with gastroenteritis in individuals exposed to contaminated waters (Aarestrup 2002; Heaney 2009; Heaney 2012; US EPA 1984). In an effort to mitigate exposure to fecal pathogens, the US EPA recommends use of *Enterococcus* for assessing marine surface waters and *E. coli* or *Enterococcus* for freshwaters, using either culture based methods or qPCR (US EPA 1986; US EPA 2012).

FIB transported via agricultural and urban runoff can be deposited in streams, where they can persist in the water column (Anderson 2005; Stumpf 2010). Furthermore, fecal bacteria are able to utilize sediments and aquatic macrophytes as refugia allowing for their release back into the water column long after the original terrestrial contamination (Badgley 2010; Badgley 2011).

While originally not thought to be a human health concern some species of FIB, such as vancomycin resistant enterococci, are now recognized as important nosocomial pathogens (Arias 2012). The risk for infection by FIB in water is a real possibility as virulence factors and antibiotic resistance genes have both been detected in enterococci

isolated from surface and drinking water (De Niederhäusern 2013; Schwartz 2003). One method to mitigate human morbidity and mortality is to reduce the introduction of FIB and other pathogens from livestock into waterways.

Considerable research has been conducted on the efficacy of creating buffers and reservoirs to prevent runoff into waterways as a mechanism to reduce fecal bacteria contamination (Coyne 1995; Coyne 1998; Jamieson 2002; Malard 1994; Mallin 2002; Stout 2005; Sullivan 2007). For example, vegetated buffers between agricultural lands and surface water have been found to decrease the load of bacteria entering the water by 98-99% (Coyne 1995; Coyne 1998; Sullivan 2007). However, the buffers do not lessen FIB entry to surface waters sufficiently for the water to subsequently be considered safe (Coyne 1995; Coyne 1998). On-farm practices such as tillage also have the ability to minimize the levels of fecal bacteria reaching surface waters (Entry 2010; Gagliardi 2000). Early work demonstrated that the decay rate of FIB in soils is the primary factor determining fecal bacteria transport into surface waters (Reddy 1981). In view of the challenges at the field and riparian steps, the best strategy for reducing bacterial loads in manure may be to treat the manure before it reaches the field.

Composting, deep stacking and windrowing have been widely adopted as industry standards to reduce pathogens in livestock waste (Chaudhry 1998; Imbeah 1998; Kelleher 2002). However, not all of these are effective in reducing risks associated with fecal contamination. Graham (2009) found that enterococci and antibiotic resistance determinants could persist in untreated, stored chicken litter for up to 120 days, demonstrating that storage alone is not a sufficient method to reduce bacterial loads to a safe level. The addition of alum, a chemical used to reduce phosphorous solubility and

ammonia volatilization, has been shown to reduce many types of bacteria in litter (Rothrock 2008). Most studies pertaining to the survival of indicator organisms in livestock wastes focus on after treatment effects (Chen 2014; Entry 2005; Sobratee 2008; Wichuk 2007). Various composting procedures such as aerating the compost and ensuring that internal temperatures exceed 55°C have been shown to be effective at eliminating pathogens (Bernal 2009; Hartel 2000; Tiquia 2002; Wichuk 2007). One challenge is that due to the heterogeneous nature of the compost heap, treatment often does not succeed in reducing the bacterial load to zero, with some pathogens surviving at low levels, despite implementing the above practices (Chen 2014; Hutchison 2005b; Macklin 2006, 2008; Shepherd 2010). In addition, after composting of poultry litter is complete, bacteria may regrow (Wilkinson 2011). Therefore, even treated litter amendments can introduce pathogens to soil.

The persistence and regrowth of fecal bacteria in poultry litter may account for the high level of these bacteria detected in agricultural runoff (Edwards 1994; Jenkins 2006; Soupir 2006). This hypothesis is supported by the fact that *E. coli* from livestock waste have been found to regrow once added to soil (Anderson 2005; Van Donsel 1967; Howell 1996). Once manure has been added to soil it is unclear how long FIB will persist; estimates range widely, from a few days to seven months probably due to variable soil edaphic properties and environmental conditions (Brooks 2009, Jenkins 2006; Lau 2001; Mishra 2008). For example, factors such as texture and depth in the soil column have both been shown to contribute to survival of bacteria in soil (Cools 2001; Stocker 2015). Furthermore, there is evidence of seasonal variation in bacterial survival in manure-amended soils (Lau 2001). This is probably due to ambient temperature and

soil moisture, which seem to be the overriding factors in the survival of fecal bacteria (Cools 2001; Desmarais 2002; Gerba 1975; Reddy 1981).

The survival of *E. coli* and fecal coliforms in livestock waste has received more attention than that of enterococci, as the former are FIBs most routinely monitored for in freshwaters (Nicholson 2005; Himathoongkham 2000). However, *Enterococcus* spp. are a genus of bacteria that are part of the normal intestinal flora of mammals and birds. The genus *Enterococcus* was originally characterized using the species *E. faecium* and *E. faecalis* (Schleifer 1984). Enterococci are Gram-positive, catalase-negative, facultative anaerobic cocci, which can grow at temperatures between 10 and 45^{o}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 present the Lancefield group D antigen (Facklam 2002). However, there are some notable exceptions to these general characteristics. Several species, including *E. durans*, *E. cecorum*, and *E. saccharolyticus* do not react with Lancefield group D antisera; *E. cecorum*, and *E. columbae* do not grow at 5°C, while *E. sulfureus*, and *E. dispar* do not grow at 45°C (Devriese 1993). Enterococci have the ability to utilize a variety of carbohydrates which can assist in phenotypic identification of individual species (Devriese 2006; Huycke 2002).

Increasing levels of antibiotic resistance in bacteria can be attributed to the use and misuse of antimicrobial agents both in medicine and agriculture. Approximately 258 million prescriptions for antibiotics were written in 2010 (Hicks 2013). Animal husbandry practices account for the largest proportion of antibiotics used in agriculture, with the total quantity administered to livestock animals being approximately 4 times greater than that given to humans. (US FDA 2013; US FDA 2015). There is a strong

relationship between antibiotics used in animal husbandry and the levels of antibiotic resistance of bacteria found in the manure of animals (Peak 2007). As much as 30-90% of all veterinary antibiotics are excreted in manure (Sarmah 2006). Locations receiving high levels of antibiotics and their metabolites have higher rates of antibiotic resistance (Zhang 2015). Furthermore, the presence of specific antibiotics in manure, such as tetracycline, has been shown to select for resistance genes specific to that antibiotic (Schwaiger 2009).

In the 1940s, researchers first demonstrated that antibiotics administered to healthy chicks could increase production weight (Moore 1946). Shortly afterwards, several researchers found that the addition of antibiotic-producing fungi to feed was more effective than their current growth promoter, B12, at increasing the weight of chickens (Hill 1950; Sunde 1951). In 1951 the United States Food and Drug Administration approved the use of antibiotics in agriculture without a prescription (Jones 2003). This change allowed poultry and livestock farmers to give sub-therapeutic levels of antibiotics to their animals regardless of their health, increasing growth rate and production in livestock. That same year there were reports of resistant bacteria in turkey poults (young turkeys) that were fed a growth promoting dosage of streptomycin (Starr 1951).

Enterococcus spp. can exhibit a wide range of phenotypic and genotypic antibiotic resistance traits. They are particularly well suited for surviving in environments that have high levels of antibiotics such as hospitals because many species are intrinsically resistant to several antibiotics (Mundy 2000; Van Tyne 2014). Additionally, the enterococci are adept at incorporating and transmitting exogenous genetic elements such as plasmids and transposons, which often contain antibiotic resistance genes (Arias 2012; Palmer 2010; Van Tyne 2014). Consequently, the enterococci are now considered

important nosocomial pathogens (Arias 2012). Furthermore, the use of avoparcin, a glycopeptide, in animal husbandry in Europe lead to increased incidence of vancomycin resistant enterococci (VRE) on poultry farms, and in non-hospitalized carriers of VRE (Aarestrup 2001; Borgen 2000; Heuer 2002; van den Bogaard 1997).

The transmission of resistant bacteria from poultry to humans has been documented in several studies (Levy 1976; Ojeniyi 1985; Ojeniyi 1989). More specifically it has been shown that poultry farmers can share resistant fecal bacteria with the birds they raise (Bass 1999; Stobberingh 1999; van den Bogaard 2001; van den Bogaard 2002). In addition, antibiotic resistant fecal bacteria have been isolated from poultry products (Klibi 2012; Vignaroli 2011).

Recently, consumers have driven the market to offer more antibiotic-free and organic products (O'Donovan 2002; Yiridoe 2005). However, it is unclear whether these practices improve safety, as there is no clear consensus from the various studies performed (Van Loo 2012). The voluntary removal of antibiotics from farms can lead to a reduction in the prevalence of resistance to specific antibiotics as well as a reduction in multidrug resistant bacteria (Sapkota 2011). However, there can be legacy effects that persist for years after ceasing to administer antibiotics to livestock, including the persistence of clinically important organisms such as vancomycin resistant enterococci (Sørum 2006).

There is a notable lack of information on the species composition of enterococci in turkey litter and the turkey gut. Likewise, the role that temperature and moisture content play in the survival of enterococci in turkey litter has yet to be defined. While some research has been performed on the survival of *E. coli* in the litter environment,

understanding how temperature and moisture content affect the survival of enterococci will help provide a more complete understanding of the potential for poultry litter to provide contaminants to surface water (Chen 2014; Wilkinson 2011). Some studies have shown a reduction in antibiotic resistant fecal bacteria and pathogens on farms that use organic practices rather than conventional practices (Sapkota 2011; Sapkota 2014). However, to date there have been no longitudinal studies performed on farms that transitioned from conventional to organic rearing practices. Understanding how the antibiotic resistance patterns of fecal bacteria change on farms transitioning to organic, antibiotic free, practices will help provide a more complete understanding of how rearing practices influence the antibiotic resistance of litter borne microbes. This study aims to provide policy makers and farmers with information integral to implementing best-management practices for disposal of used poultry litter.

Research Questions

1. Are enterococci present in fresh or used litter on turkey farms?
2. What are common species of enterococci in the litter of organically and conventionally raised turkeys?
3. Is there a difference in the antibiotic resistance profile of enterococci isolated from the litter of organically and conventionally raised birds?
4. Does the amount of moisture in turkey litter affect survival and persistence of *E. faecium*?
5. Does ambient air temperature (5°C, 30°C) affect survival and persistence of *Enterococcus* in litter?

Methods

Study Area

The study site was a turkey farm in Shenandoah Co, VA. The farm contained four poultry houses from which all samples were collected. Each house was divided into two parts. One end (50' wide by 180' long) was exclusively used for the first 4-6 weeks of rearing; it had cement floors and new wood shavings were brought in as bedding for each flock. The used bedding was moved to the other 'finishing' end of the house when the birds were transferred there after 4-6 weeks. The finishing end measured 50' wide by 525' long; It had dirt floors and the bedding was changed after every three flocks. Each flock cohort comprised between 7,000 and 9,000 turkey poults. The poults at the farm are a hybrid breed from a cross between the British United Turkey and the Nicholas Turkey (Personal Communication, Virginia Poultry Growers Co-operative 2013).

During the period of the study the farm transitioned from conventional practices including administration of the antibiotic oxytetracycline prophylactically, to an organic, antibiotic free, practice. The first litter sample collections (July 2013) were taken from a house in which the birds were raised following conventional practices, the second (October 2013) during growth of birds without antibiotic treatments. The grow house was cleaned between the two cohorts.

Field Sampling

Fresh wood shavings were sampled on two occasions (07/02/2013 and 10/30/2013), by collecting from the top five cm of the new, unused bedding prior to addition of the birds. Six composite samples were created using systematic random area sampling. The poultry house was divided using a grid to create three rows with each row

containing 14 blocks. One sample was collected from each row by randomly selecting three blocks in each of the three rows. One sample was created by randomly selecting three blocks from each outer column and combining them. The last two samples were collected by randomly selecting three blocks from each row and combining litter from all three columns. The composite samples were collected in sterile five gallon buckets, filled no more than half way. Each was mixed by vigorous shaking for 30 seconds and was then transferred into a one-gallon Whirl-Pak® bag which was filled half full, sealed, and placed on ice for transport to the laboratory.

At the end of the two turkey grow cycles (17-19 weeks), used litter was collected. For the first sampling event on 07/02/2013 (conventional practice), three flocks of birds had been raised on the litter. The litter had been windrowed between flocks where it was turned three times, each successive turning occurred 72 hours after the previous turning and then spread. Litter collection followed the third flock. The litter had not been windrowed and was collected from the hard packed floor following the same sampling scheme, but using 52 blocks per row instead of 14. The top 5 cm was loosened using a sterilized hand tiller and garden spade that were disinfected with Sporicidin®, collected in bulk using the garden spade and placed in five gallon buckets that had been washed with a 10% bleach solution. Lids were snapped into place for transportation and stored at room temperature for use in microcosm experiments.

On the second sampling date, 10/30/2013 (organic practice), with only one flock of turkeys raised on the litter, the litter had been stacked the previous day in three windrows approximately one-meter high. The litter was loose and easily collected; it had

only been used for one flock. These samples were collected using the same sampling scheme used to collect on 07/02/2013.

Processing of samples from the farm

In the laboratory, subsamples were taken from each litter sample after thoroughly mixing samples within the Whirl-Pak® bags. Bacteria were then extracted from each subsample by combining 10 g of litter in a plastic stomacher bag with 95 mL of sterile phosphate-buffered saline (PBS) (112.1 mg Na_2HPO_4, 23.75 mg NaH_2PO_4 $2H_2O$, 4.25 g NaCl, and 95 mL of deionized H_2O) (Zuberer 1994). A separate 10 g of litter from each composite sample were weighed in an aluminum weigh boat and placed in a drying oven at 105^{o}C for 24 hours to determine dry weight/wet weight ratio. Each stomacher bag was agitated in an Interscience BagMixer® for 2 minutes; then it was removed and gently squeezed to separate any aggregates of litter. This was repeated twice more for each sample. After the third agitation the bags were sealed and mixed by shaking by hand for 20 seconds and 1 ml of supernatant was removed and added to sterile PBS for serial dilutions. Serial dilutions were performed to 10^{-9}. A 100 µL aliquot of diluent was spread on either Tryptic Soy Agar (TSA) (236950; Becton, Dickinson and Company, Sparks, MD) or membrane-Enterococcus Indoxyl-β-D-Glucoside Agar (mEI) (215047; Becton, Dickinson and Company, Sparks, MD) plates. TSA plates were incubated at 35^{o}C for 24 hours ± 2 hours and mEI plates were incubated at 41^{o}C for 24 hours ± 2 hours. Total culturable aerobic bacteria were quantified by counting all colonies on the TSA plates using 2-5x magnification and overhead lighting from a white fluorescent light, while total enterococci were quantified by counting all blue, purple, and pink colonies on mEI plates following EPA method 1600 (US EPA 2006).

Litter collected on 07/02/13 and 10/30/13 yielded presumptive enterococci (blue, pink, and purple colonies) on mEI plates. These colonies were re-streaked onto mEI plates and incubated for 24 h at 35°C. Pure cultures were stored on TSA slants at 4°C.

Species Identification

Carbon source utilization, a phenotypic fingerprinting method, was used to identify species of *Enterococcus* isolated from the litters using the Biolog System, following the manufacturer's instructions (71102; Biolog, Inc., Hayward, CA). Isolates were transferred to Biolog Universal Growth Medium supplemented with 5% Sheep's Blood (BUG^{TM}+B) (71102; Biolog, Inc., Hayward, CA) and incubated at 33°C for 18-24 h. Suspensions of each isolate were transferred into inoculating fluid-A (IF-A) (72401; Biolog, Inc., Hayward, CA) using a sterile swab (3321; Biolog, Inc., Hayward, CA) to achieve a transmittance between 90-98% (T) ± 2% at 590 nm in the Biolog turbidimeter (3531; Biolog, Inc., Hayward, CA). The resulting suspension was then pipetted into a 96-well Biolog GEN III MicroPlate (1030; Biolog, Inc., Hayward, CA), and plates were incubated for 20-28 h at 33°C. After incubation, plates were read using the MicroLog Microbial Identification System 3, (Release 5.2; Biolog, Inc., Hayward, CA) (Biolog, 2004) to obtain color intensity and +/- well reactions. Isolates that did not confirm to species level identification using the automated plate reader were also read manually to ensure correct identification.

Confirmed *Enterococcus spp.* isolates were stored in cryogenic storage vials at -80°C (2 ml, 66008-284; VWR, West Chester, PA). After incubation in TSB, 600 µl of the bacterial culture was mixed with 400 µl of a 50/50 combination of TSB and sterile glycerol. Duplicate vials were made of each isolate.

Microcosm Experiments

The survival of enterococci in turkey litter under various conditions was evaluated using turkey litter microcosm experiments. The independent variables manipulated in these experiments were temperature and moisture content (Figure 1). All experiments were conducted using the American Type Culture Collection (ATCC) 19434 strain of *E. faecium*.

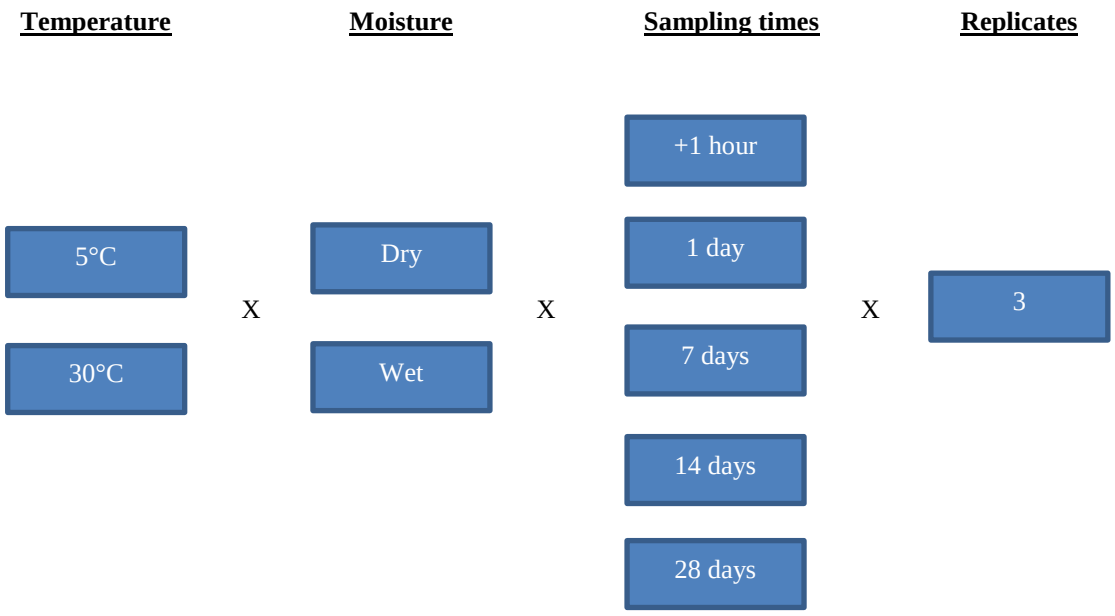

Figure 1: Full factorial experimental design for *E. faecium* survival experiments. Each temperature was paired with each moisture (Dry: ≤ 10% H_20; Wet: 35-40% H_20). Resulting temperature × moisture combinations were sampled at five time points (1 hour, 1 day, 7 days, 14 days, and 28 days) with three replicates per each treatment for a total of 15 microcosms per treatment.

Experiments were conducted at incubation temperatures of 5°C and 30°C to represent the high (summer) and low (winter) average temperatures in the Shenandoah Valley. Two moisture levels were used to simulate dry and wet conditions. Dry litter contained less than 10% water, while wet litter contained between 30% and 40% w/v water.

Experiment 1 consisted of 50 mL VWR light sensitive centrifuge tubes of autoclaved wet and dry litter inoculated with *E. faecium* which were incubated at 5°C. Experiment 2 was the same design, except that incubation was at 30°C. Each experiment was performed twice. Each experiment consisted of 40 litter microcosms (50 mL centrifuge tubes), 15 containing wet litter and 15 with dry. The remaining 10 centrifuge tubes were controls, consisting of un-inoculated litter - five centrifuge tubes of wet litter and five of dry litter.

To prepare the bacterial culture BD Difco™ tryptic soy broth (TSB) (211825; Becton, Dickinson and Company, Sparks, MD) (5 mL) was inoculated with a pure culture of the ATCC 19434 strain of *E. faecium*, and incubated at 35°C in a culture rotator overnight, prior to creating litter microcosms. After incubation, two 1 mL aliquots of broth culture were centrifuged at 5000 RPM for 3 minutes, the supernatants were decanted and the cells were re-suspended in 1 mL of sterile PBS. The cells were washed a total of three times with PBS. The final cell suspension was added to sterile PBS dropwise and vortexed to mix. The optical density of the resulting solution was measured using a spectrophotometer, with the wavelength set at 625 nm and un-inoculated PBS as a blank. Once the PBS cell suspension reached an optical density between 0.080 and 0.100, 3 mL was added to 37 mL of sterile PBS in a 250 mL culture bottle.

The litter for the microcosms was prepared by sieving bedding from the end of the conventional grow cycle through 0.25” mesh to exclude large aggregates. The sieved material was placed into metal trays to a depth of ~2 cm, covered with aluminum foil and autoclaved using a 60-minute gravity cycle. The cycle was repeated three days later. Once the litter had been autoclaved twice it was mixed with other autoclaved batches of

litter to make a composite sample. A subset of litter was removed, dried overnight in an oven at 105°C and autoclaved a third time, again using a 60-minute gravity cycle. Experiments were begun within one week of the final autoclaving. Litter was added to each centrifuge tube using a sterile scoop. A separate sample of litter was dried at 105°C for 24 hours to determine dry weight/wet weight ratios.

A subsample of sieved litter before autoclaving and a subsample of litter that had undergone three autoclave cycles was sent to the Pennsylvania State University Agricultural Analytical Services lab for analyses to determine percent solids, total nitrogen (N), Ammonium N (NH_4-N), Organic N, Total Phosphate (P_2O_5), and total Potash (K_2O) (Appendix 1).

Dry Litter

One scoop of litter (~2 g), was added to each of the 15 centrifuge tubes. These microcosms were then inoculated with the prepared *E. faecium* (0.5 mL) culture. Another scoop of litter was added; then the tube was capped and shaken by hand 20 times. This process was repeated a second time and the microcosm was topped off with litter to bring the total mass to 16 g (litter and inoculum). Finally, each microcosm was shaken 20 times to disperse bacteria throughout the microcosm. An additional five control litter microcosms were created with sterile PBS in place of the *E. faecium* inoculum.

Wet Litter

One scoop of litter was added to each of the 15 centrifuge tubes. These litter microcosms were then inoculated with the prepared *E. faecium* (0.5 mL) culture. Another scoop of litter was added to each tube. This was followed by adding 2 mL of sterile Millipore water, and then the tubes were capped and shaken by hand 20 times. This

process was repeated a second time, except 2.5 mL of sterile Millipore water were added, and the microcosm was topped off with litter to bring the total mass to 16 g (litter, water and inoculum). After completion, each microcosm was shaken 20 times to disperse bacteria throughout the microcosm. An additional five control litter microcosms were created with sterile PBS in place of the *E. faecium* inoculum.

Incubation/ Extraction/ Filtration

Litter microcosms were incubated at one of two temperatures (5°C or 30°C). Three microcosms of each treatment (wet, dry) and the corresponding control, for a total of four microcosms per experimental treatment, were removed from the incubator at specific time points, (+1 hour, 1 day, 7 days, 14 days, and 28 days). Before transferring the contents of each microcosm into a shaker flask filled with 500 mL sterile PBS, 50 mL of the PBS was decanted into a sterile 50 mL conical tube. Each litter microcosm was then poured into a separate shaker flask. The litter microcosm container was rinsed up to six times with PBS from the conical tube until no litter remained in the litter microcosm. The remainder, if any, of the PBS used for rinsing was poured back into the original shaker flask so that the total amount of PBS and litter in the shaker flask was 500 ml and 16 g respectively. This procedure was used for each microcosm. Using a Burrell Wrist Action Shaker™ (75-775-24; Model 75, Pittsburgh, PA), samples were shaken in the 1 L flasks for one hour (Zuberer 1994). Flasks were then removed and left to stand undisturbed for 30 to 60 minutes to allow the litter to settle out of solution. Following this, supernatant was extracted to create a serial dilution series in milk dilution bottles. The diluent was filtered following EPA Method 1600: Enterococci in Water by Membrane Filtration using membrane-Enterococcus Indoxyl-β-D-Glucoside Agar (mEI),

with the exception that PBS was used to rinse (US EPA 2006; Zuberer 1994). Blue-haloed colonies were counted after 24 h ± 2 h incubation at 41°C to determine numbers of enterococci in each sample.

Antibiotic Resistance Analysis

All isolates from mEI samples collected on 07/02/2013 and 10/30/2013 which were identified as members of the genus *Enterococcus* were analyzed for antibiotic resistance. Pure cultures were transferred from TSA slants onto TSA plates. Cultures were streaked for isolation, and up to four isolated colonies of each isolate were selected from the TSA plate to inoculate 5 ml tubes of prepared TSB. After six hours of incubation, inoculated TSB suspension was added to blanked TSB-filled cuvettes until absorbency was between 0.08 and 0.10 at 625 nm. Once proper absorbency was achieved, each suspension was plated using a triple-lawn streak onto two Mueller Hinton Agar I (90006-573, Becton, Dickinson and Company, Sparks, MD) plates utilizing the standardized Kirby Bauer Disk Diffusion method (Clinical and Laboratory Standards Institute 2006a, 2006b, 2008, 2012). A panel of twelve different antibiotics, as commercially prepared discs (BD BBL Sensi-Disc Antibiotics; Becton, Dickinson and Company, Sparks, MD) (Table 1) was dispensed onto a plate, the plates were incubated for 18-24 h at 35°C. Antibiotics were selected based on the suggested grouping of antimicrobial agents and interpretive criteria for disk diffusion and dilution susceptibility testing for *Enterococcus* species according to the Clinical and Laboratory Standards Institute (CLSI) (CLSI 2014). Additionally, antibiotics were selected to include representatives from different groups of antibiotics and different uses among various animals. Diameters of zones of inhibition were measured in mm along with susceptibility

(S), intermediate (I), and resistant (R) patterns (S-I-R patterns) (Table 1), through automated image analysis by the Biomic™ plate reader which uses standard CLSI zone diameter information to categorize isolates into S/I/R. (Giles Scientific Inc., Santa Barbara, CA). This also ensured uniformity for future comparisons with *Enterococcus* isolates from unknown sources.

Table 1: List of antibiotics used to develop Antibiotic Resistance Profiles for *Enterococcus* isolates. Susceptible (S), Intermediate (I), and Resistant (R) zones of inhibition ranges (mm) for *Enterococcus* are based on CLSI standards (2014) and incorporated into Biomic™ plate reader software (CLSI 2014).

Antibiotic	**Abbreviation**	**Concentration**	**S**	**I**	**R**
Ampicillin	AM	10 µg	≥17	-	≤16
Chloramphenicol	C	30 µg	≥18	13-17	≤12
Ciprofloxacin	CIP	5 µg	≥21	16-20	≤15
Doxycycline	D	30 mg	≥16	13-15	≤12
Erythromycin	E	15 µg	≥23	14-22	≤13
Gentamicin	GM	10 µg	≥15	13-14	≤12
Linezolid	LZD	30 µg	≥23	21-22	≤20
Quinupristin/ Dalfopristin	SYN	15 µg	≥19	16-18	≤15
Rifampin	Ra	5 µg	≥20	17-19	≤16
Streptomycin	S	10 µg	≥15	12-14	≤11
Tetracycline	Te	30 µg	≥19	15-18	≤14
Vancomycin	V	30 µg	≥17	15-16	≤14

Data Analyses

Enterococci levels were reported in colony forming units per gram dry weight (CFU/gdw) using the formula:

$$\text{CFU/ } g_{DW} = \frac{\text{colony counts}}{\text{dilution factor}} \text{ x LDF x } \frac{1}{\text{calculated dry weight}^*}$$

*calculated dry weight =C-A/B-A

where LDF (litter dilution factor) = 10.5 (This is unitless and indicates that 10.5 mL of diluent is displaced per gram of litter) A = empty dish weight (g), B = Dish + litter (g), and C = Dish + litter after drying (g) (Zuberer 1994). The DF was determined based on 10g of dry litter displacing approximately 25 mL of buffer making total volume of the litter/buffer mixture 525 mL; so the calculation for the DF is (525 mL/ 50 gdw).

Statistical Analyses

Each microcosm experiment was evaluated to determine a) if there was any growth, a significant increase in the mean CFU/gdw of enterococci in the microcosms between any two time points and b) if there was decay, a significant decrease in the mean CFU/gdw of enterococci in the microcosms between any two time points. This was determined by comparing means using a two sample t-test. Each of the above analyses were carried out using R version 3.8.2. A linear mixed effects model was used to compare each treatment in the microcosm experiments. The model specified temperature, moisture, sampling day, and all 2- and 3-way interactions as fixed effects and random effects were specified as the individual replicated experiments nested within each temperature and moisture combination, the combination of sampling day and individual replicated experiment nested within each temperature and moisture combination and a default residual random error. Estimate statements were written to compare the interaction between the main effects temperature and moisture on each sampling day. Log_{10} transformed data were used to meet normal distribution and homoscedascity. The Kenward and Roger (1997) method was used for determining degrees of freedom.

Chi-square analysis was conducted to compare proportions of isolates resistant to each of the 12 antibiotics. This analysis was performed with resistant and intermediate isolates combined and with resistant isolates alone. The isolates from each litter source were aggregated and mean of antibiotic resistant phenotypes (not including intermediate phenotypes) was compared using the Wilcoxon-t test.

Quality Assurance/ Quality Control

Quality control samples were run (i.e. positive controls, negative controls, and blanks) for each selective medium lot, as well as positive controls and sterility checks for all batches of media prepared. Positive and negative control cultures for mEI and mE agars were *E. faecalis* ATCC 19433 (positive) and *Escherichia coli* ATCC 11775 (negative). Media log sheets indicating date, medium, volume, pH, and lot numbers were kept for all prepared media. All inoculated plates, tubes, broths etc. were autoclaved in biohazard bags with indicator tape for at least 30 min at 121°C prior to disposal. Media that supported the growth of negative controls, did not support the growth of positive control, failed sterility checks, or failed pH values were discarded and remade.

Quality controls for carbon source utilization were followed according to the protocol described in the MicroLog™ System Release 4.0 User Guide (Biolog 1999). Each lot of BUG/B and GP2 MicroPlates™ had been tested for internal quality control standards before being released for sale. Internal quality controls for BUG™+B conducted by Gibson Laboratories, LLC., tested gel strength, bioburden performance, pH (7.3 ± 0.1), and biological performance utilizing *Streptococcus pyogenes* ATCC 19615, *Streptococcus pneumoniae* ATCC 6305, *Staphylococcus aureus* ATCC 25923, and *Escherichia coli* ATCC 25922 (Gibson Laboratories, LLC, Lexington, KY). Following

BIOLOG recommendations, a set of four control strains, 2 Gram-negative, and 2 Gram-positive organisms, were streaked onto BUG/B plates, inoculated onto GEN III MicroPlates™, and analyzed via the Biolog MicroStation™ Reader for quality control purposes. These strains were *Escherichia coli* ATCC 11775, *Paenibacillus polymyxa* ATCC 842, *Staphylococcus epidermidis* ATCC 12228, and *Stenotrophomonas maltophilia* ATCC 13637.

Laboratory Duplicates

All analyses (quantification, species identification, and membrane filtration) were performed with one duplicate for each treatment at each filtration event (1 hour, 1 day, 7 days, 14 days, 28 days) during the experiment. Each survival experiment was conducted twice.

Results

Field Study

In order to determine if enterococci were present in turkey litter, total culturable aerobes and enterococci were quantified in fresh bedding and in used litter. Technical replicates were collected at each sampling date; however since all samples came from the same house, statistical analysis and variability were not calculated to avoid pseudo-replication. Fresh bedding contained 2.36×10^3 CFU/gdw total culturable aerobes on the first sample date while enterococci were below the detectable limit; the total culturable aerobes were slightly higher at the second sampling, 3.98×10^4 CFU/gdw; however, enterococci remained undetectable. Used litter contained appreciably higher total culturable aerobes at each sampling date, 7.96×10^8 CFU/gdw and 2.72×10^8 CFU/gdw respectively; Enterococci data were only obtained for the second

used litter sample collection (10/30/2013) and were present at concentrations of 1.39×10^5 CFU/gdw.

E. faecium was the most common species of *Enterococcus* identified from the 99 isolates obtained from the litter of conventionally raised turkeys, and 53 isolates obtained from organically raised turkeys (Table 2). *E. durans* and *E. faecalis* comprised a higher proportion of isolates collected from the litter of conventionally, than organically, raised birds (Table 2). The litter of organically raised birds contained a higher prevalence of *E. pseudoavium* and *E. asini* (Table 2). There were a total of eight different species of *Enterococcus* identified, of which two were only found in litter from conventionally raised birds (*E. ratti* and *E. gallinarum*) and one was only found in the litter of organically raised birds (*E. mundtii*) (Table 2).

Table 2: Identified *Enterococcus* spp.. Values reported are the percentage of *Enterococcus* spp. isolated from the litter of conventionally (n=99) and organically (n=53) raised turkeys.

Species	**Conventional**	**Organic**
E. faecium	43.3	54.7
E. durans	29.9	9.4
E. faecalis	18.5	9.4
E. pseudoavium	3.1	11.3
E. ratti	2.1	0.0
E. gallinarum	2.1	0.0
E. asini	1.0	13.2
E. mundtii	0.0	1.9

Antibiotic resistance

The proportion of isolates resistant to each antibiotic was viewed in aggregate. Of the 12 antibiotics tested, the proportion of isolates resistant to six of those antibiotics (ampicillin, chloramphenicol, linezolid, quinipristin/dalfopristin, rifampin, and vancomycin) was not significantly different between farming practices (Appendix 2). The proportion of resistant isolates from the litter of organically raised turkeys was significantly lower for tetracycline, doxycycline, and gentamicin than from the conventionally raised turkey litter (both resistant alone, and resistant and intermediate isolates combined, for all three antibiotics) (Figure 2). Conversely, the proportion of isolates resistant to streptomycin (both resistant alone, and resistant and intermediate isolates combined), erythromycin (only when resistant and intermediate isolates combined), and ciprofloxacin (only resistant alone) was significantly higher in the litter of organically raised turkeys (Figure 3).

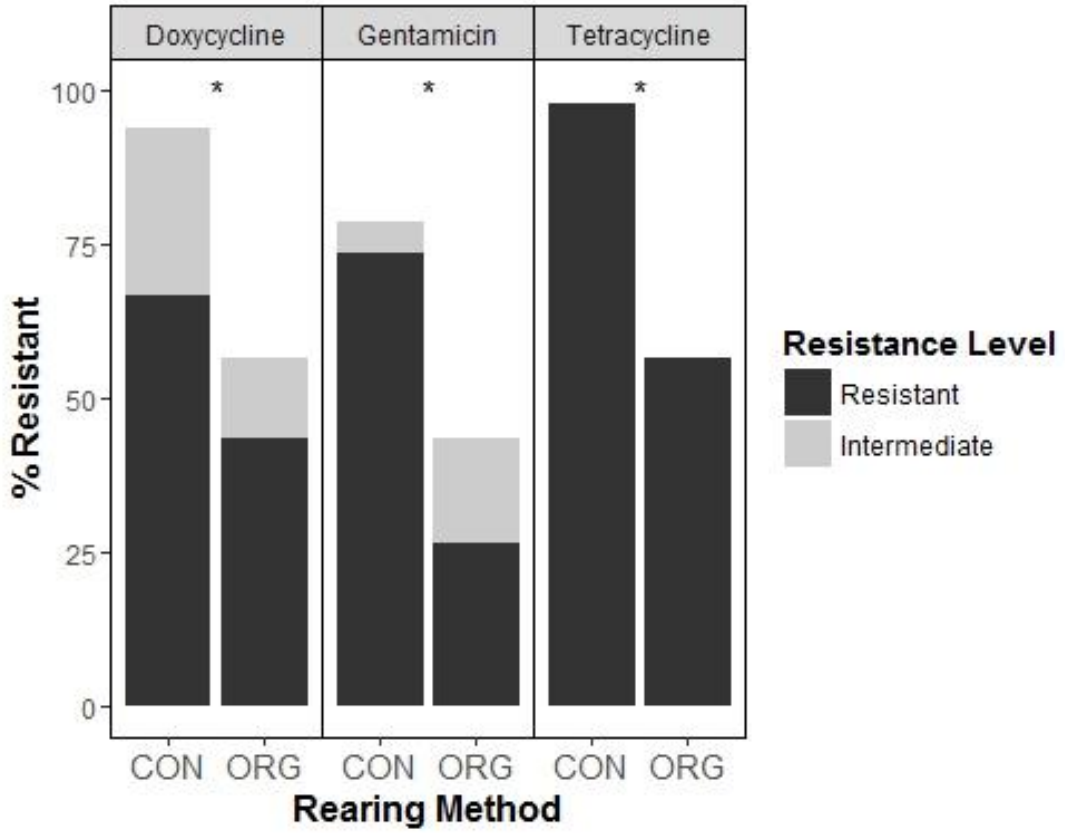

Figure 2: Resistance to tetracycline, doxycycline, and gentamicin of enterococci isolated from litter used in conventional (CON) vs. organic (ORG) rearing.
*Significant difference between treatments when comparing proportions of resistant isolates and proportion of resistant+intermediate isolates; $p<0.05$

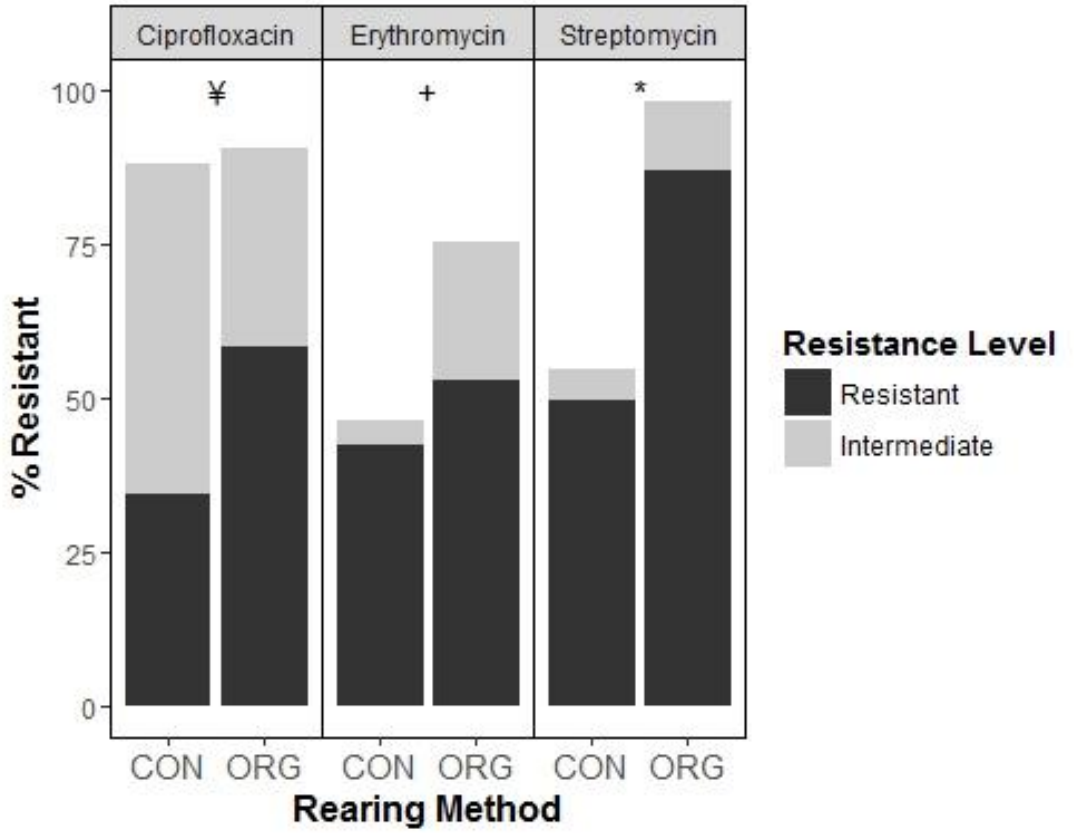

Figure 3: Resistance to streptomycin, erythromycin, and ciprofloxacin of enterococci isolated from litter used in conventional (CON) vs. organic (ORG) practice.
* Significant difference between treatments when comparing resistant isolates, not resistant when comparing resistant+intermediate isolates; $p<0.05$.
+ Significant difference between treatments when comparing resistant+intermediate isolates, not significant when comparing only resistant isolates; $p<0.05$.
¥ Significant difference between treatments when comparing proportions of resistant isolates and proportion of resistant+intermediate isolates; $p<0.05$.

Isolates from the litter of conventionally raised turkeys were resistant to more antibiotics than isolates from the litter of organically raised turkeys according to the Wilcoxon test ($W=3243.5$; $p=0.0145$) (Figure 4). When comparing resistant+intermediate isolates from the litter of conventionally raised turkeys were still resistant to more antibiotics ($W=3116$; $p=0.0499$); however, when comparing only intermediate isolates

there was no significant difference between treatments (W=2504; p=0.6276) (Appendix 2).

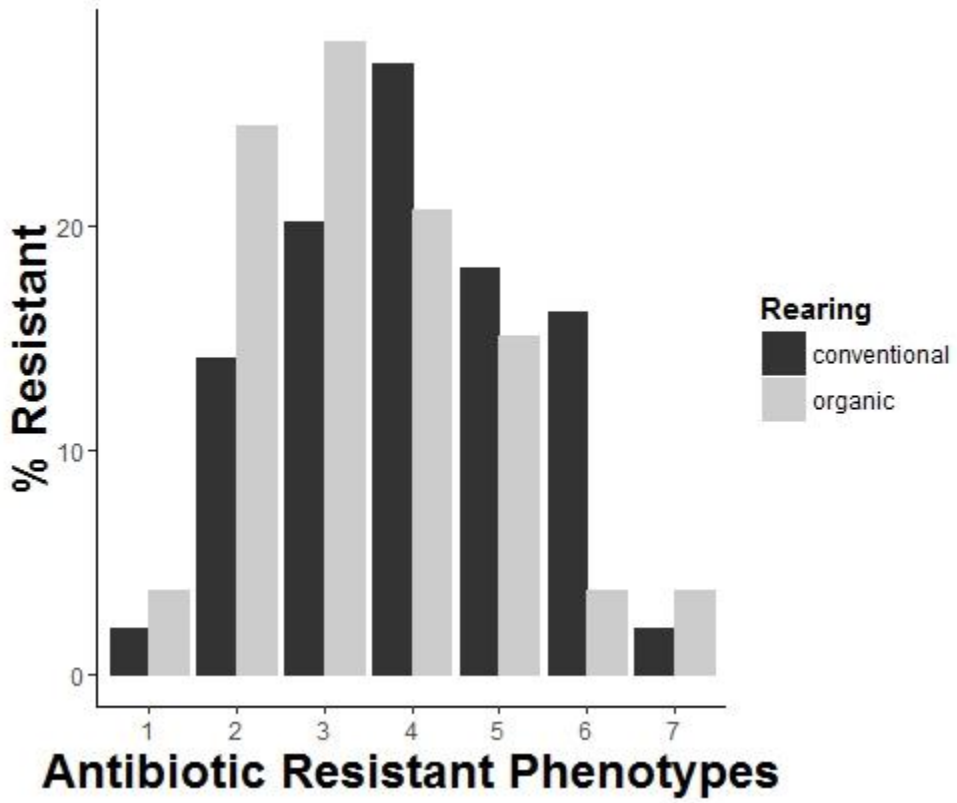

Figure 4: Isolates exhibiting resistance to one or more antibiotics. Only resistant phenotypes were included; (W=3243.5; p=0.0145).

Microcosm experiments

Survival of *E. faecium* differed by treatment (Figure 5). The four experiments (30˚ C wet, 30˚ C dry, 5˚ C wet, 5˚ C dry) were each performed twice and the treatment results in each individual experiment followed the same trend (Appendix 2). However, there was a large amount of variation between the duplicate experiments in the 30˚C dry experiment, as can be seen by the SE bars and shown in the Appendix

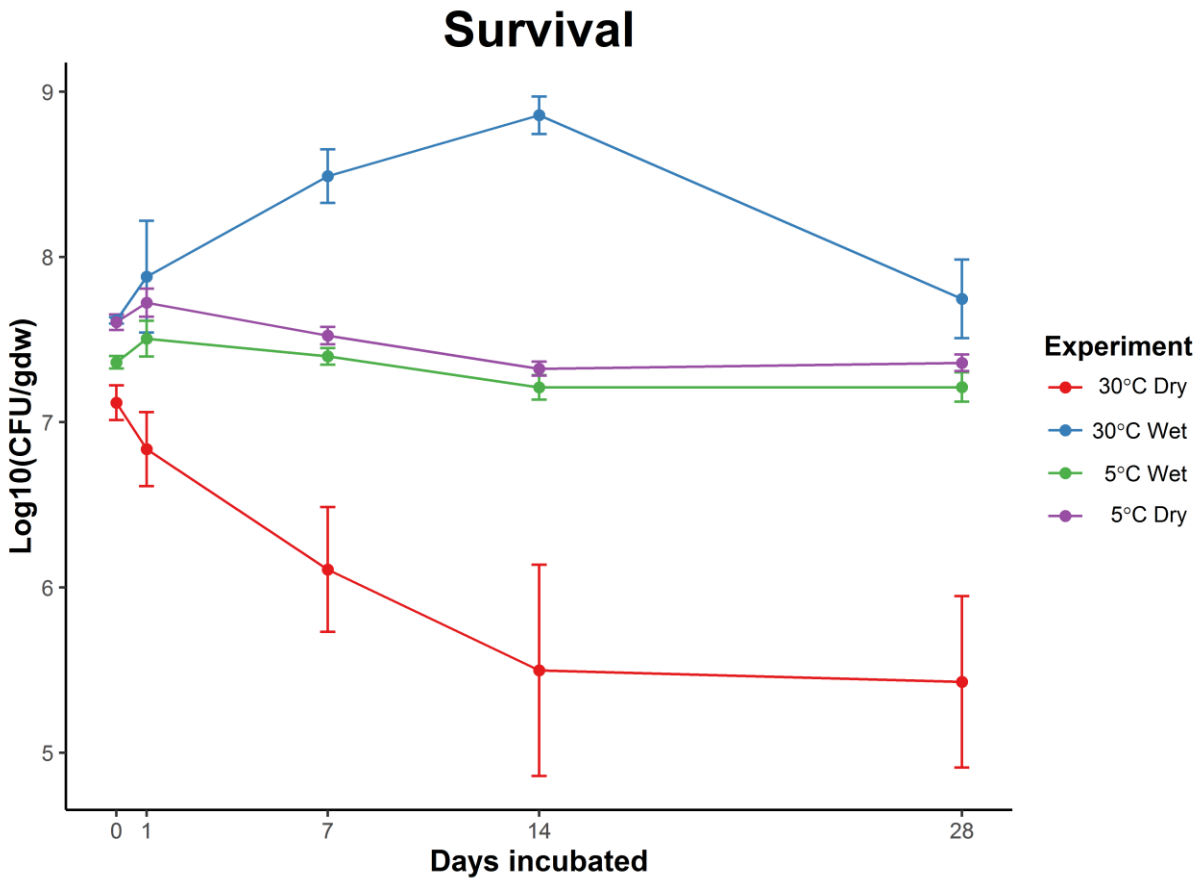

Figure 5: Survival curves of *E. faecium* in microcosms sampled over the course of 28 days. Error bars represent standard error. Each experiment was conducted twice.

Growth (i.e. increase in CFU/gdw litter) of 1.24 $\log_{10}$ (CFU/gdw) was observed for the first 14 days in the 30˚C incubated wet litter experiment, but no growth was seen in the other treatments (Figure 5; Appendix 2).

Decay (i.e. reduction in CFU/gdw litter) was observed over the duration of the experiments for both the 30˚C dry and 5˚C wet treatments. While significant decay, as compared to day 0, was first observed on day 7 in the 30˚C dry experiment, it was not observed until day 14 in the 5˚C wet experiment (Figure 5; Appendix 2). In the 30˚C dry experiment there was a reduction in the enterococci of 1.69 $\log_{10}$ (CFU/gdw) over the 28-day experiment. Although, the decrease was significant, the reduction was only 0.28 $\log_{10}$

(CFU/gdw). There was significant decay between day 14 and day 28 in the 30˚C wet experiment (Figure 5; Appendix 2). During that time frame the enterococci were reduced by 1.11 $\log_{10}$ (CFU/gdw) In contrast there was no significant reduction in the population of *E. faecium* in dry litter at 5˚C, the bacteria persisted throughout the 28-day period of the experiment (Figure 8; Appendix 2).

Survival model

A mixed effects model was used to compare the survival of *E. faecium* in each treatment (Appendix 3; Appendix 4). There were no significant interactions on day 0 or day 1 (Table 3). Additionally, there were no significant differences between the levels of main effects on day 0 and day 1 (Table 4). There was a significant interaction between temperature and moisture on days 7, 14, and 28 ($p \leq 0.1$) (Table 3). On day 7 there were significant differences in bacteria levels between the dry and wet treatments at 30˚C (Table 5). However, there were no significant differences in bacteria numbers between the levels of moisture at 5 ˚C or between the levels of temperature at either moisture level (Table 5). On day 14 there were significant differences between the dry and wet treatments at 30˚C and between the 5˚C and 30˚C treatments with dry conditions (Table 5). However, there were no significant differences between the levels of moisture at 5. ˚C or between the levels of temperature with wet moisture (Table 5). On day 28, as on day 14, there were significant differences between the dry and wet treatments at 30˚C and between the 5˚C and 30˚C treatments with dry moisture (Table 5). However, there were no significant differences between the levels of moisture at 5 ˚C or between the levels of temperature with wet moisture (Table 5).

Table 3: Estimate statements comparing the interaction between moisture and temperature on each day of the experiment. SE=Standard Error, DF= Degrees of Freedom *p ≤ 0.10.

	Estimate	SE	DF	t Value	p- value
Day 0	0.2552	0.9823	7.36	0.26	0.8021
Day 1	0.8273	0.9823	7.36	0.84	0.4262
Day 7	2.2996	0.9927	7.65	2.32	0.0506*
Day 14	3.242	0.9852	7.45	3.29	0.0122*
Day 28	2.3738	0.9855	7.46	2.41	0.0448*

Table 4: Estimate statements comparing differences within one factor (temperature or moisture) not dependent on the other factor. SE=Standard error, DF= Degrees of freedom *p ≤ 0.10.

Comparison	Day	Estimate	SE	DF	t value	p-value
Temperature (5 v 30) averaged over moisture	Day 0	0.1168	0.4912	7.36	0.24	0.8185
Moisture (Dry v Wet) averaged over temperature	Day 0	-0.3707	0.4912	7.36	-0.75	0.4738
Temperature (5 v 30) averaged over moisture	Day 1	0.255	0.4912	7.36	0.52	0.6189
Moisture (Dry v Wet) average over temperature	Day 1	-0.63	0.4912	7.36	-1.28	0.2385

Table 5: Estimate statements comparing differences among levels of one factor dependent upon a second factor. SE=Standard error, DF= Degrees of freedom *p ≤ 0.10.

Comparison	Days	Estimate	SE	DF	t Value	p-value
Temperature (5 v 30) at moisture (dry)	Day 7	1.2894	0.6873	7.06	1.88	0.1024
Temperature (5 v 30) at moisture (wet)	Day 7	-1.0102	0.7162	8.26	-1.41	0.1949
Moisture (Dry v Wet) at temperature 5	Day 7	-0.1262	0.6861	7.01	-0.18	0.8593
Moisture (Dry v Wet) at temperature 30	Day 7	-2.4258	0.7173	8.31	-3.38	0.0091*
Temperature (5 v 30) at moisture (dry)	Day 14	1.7131	0.6873	7.06	2.49	0.0412*
Temperature (5 v 30) at moisture (wet)	Day 14	-1.5289	0.7058	7.84	-2.17	0.0629*
Moisture (Dry v Wet) at temperature 5	Day 14	-0.1133	0.6861	7.01	-0.17	0.8735
Moisture (Dry v Wet) at temperature 30	Day 14	-3.3553	0.707	7.89	-4.75	0.0015*
Temperature (5 v 30) at moisture (dry)	Day 28	1.9923	0.6878	7.08	2.9	0.0228*
Temperature (5 v 30) at moisture (wet)	Day 28	-0.3815	0.7058	7.84	-0.54	0.6038
Moisture (Dry v Wet) at temperature 5	Day 28	-0.1464	0.6861	7.01	-0.21	0.8372
Moisture (Dry v Wet) at temperature 30	Day 28	-2.5202	0.7074	7.91	-3.56	0.0075*

There was no significant difference in bacteria levels due to moisture (wet or dry) at 5˚C but there were significant differences at 30˚C between moisture treatments, with bacterial survival being significantly greater in wet than dry litter. This can be attributed to the fact that growth occurred initially at 30˚C in the wet microcosms but declined to near initial inoculation levels by the end of the 28-day incubation. The bacteria levels in 30˚C dry incubations decreased steadily from the onset of the experiment.

Discussion

In this study, enterococci were isolated from the litters of turkeys reared using conventional and organic practices. Since enterococci were not found in fresh wood shavings (unused litter material) it is possible that the isolated enterococci found in the used litter were due to farmer contamination, present in the food, or present in the water. However, due to previous evidence that enterococci were harbored in the excrement of turkeys, it is most likely that the main source of enterococci in the turkey litter samples was turkey feces. The concentrations found in the turkey litter were in the same range as those found in previous studies (Brooks 2010; Schefferle 1965). Although it cannot be concluded that the enterococci grow while in the litter, Schefferle (1965) noted that in their study the concentrations of enterococci were actually higher in the litter than in the feces of the birds; however, these data were not corrected for moisture content.

The dominance of *E. faecium* in both litter types (Table 2) is consistent with previous reports of enterococci species in poultry litter and poultry intestines (Devriese 1991, Debnam 2005). *E. hirae, E.* cecorum and *E. casseliflavus* were not identified in our samples which differs from the findings of Devriese (1991) who identified *E. casseliflavus and E. hirae* in low proportions in all age groups of broiler chickens and found *E. cecorum* as the most common species in the oldest age class. This may be due to the low number of isolates identified to species in our study (Table 2). Differences in enterococci species (*i.e*. a higher proportion of *E. durans* and *E. faecalis* were collected from the litter of conventionally raised birds while a higher proportion of *E. pseudoavium* and *E. asini* were collected from the litter of organically raised birds (Table 2)) between the two sets of litter may be attributed to the treatment (i.e. organic or conventional), or

could be attributed to other differences in how the litter was handled; for example, the litter from the conventional birds had been used to grow two previous flocks of birds, and was composted in between each flock (Table 2).

There were a total of eight different species of *Enterococcus* identified in this study, of which two were only found in litter from conventionally raised birds (*E. ratti* and *E. gallinarum*) and one was only found in the litter of organically raised birds (*E. mundtii*) (Table 2). We have no evidence that *E. ratti*, *E. pseudoavium*, or *E. asini* have been isolated from poultry litter (Devriese 1991; Hayes 2004; van den Bogaard 2001). *E. ratti*, has been previously associated with enteric disorders in rats, *E. pseudoavium* has been isolated from cows with bovine mastitis, and *E. asini* was isolated from a fistulated donkey (Teixeira 2001; Collins 1989; de Vaux 1998). In contrast to our finding of *E. faecium* as the most abundant species in litter, some studies have found *E. faecalis* to be the most common species in the fresh or stored litter of birds raised using organic practices (Graham 2009, Sapkota 2011). This may be due to differences in the survival of the two species in litter, difference in litter compositions, or types of turkeys used in the other studies. Several factors such as pH, litter temperature and moisture content have been shown to be key in determining the overall community structure in broiler litter (Lovanh 2007). Although they were closely related, *E. faecium* and *E. faecalis* exhibit different growth responses and metabolic activity under a variety of pH and temperatures (Morandi 2005). Thus, physicochemical properties of different litters may account for variations in both quantities of enterococci and species distribution.

None of the enterococci isolated in this study were resistant to linezolid, chloramphenicol, or vancomycin and only a few isolates were resistant to ampicillin,

rifampin, or quinipristin/dalfopristin, other than those species (*E. faecalis*, *E. gallinarum*) that were intrinsically resistant to quinipristin/dalfopristin. Under conventional practices, the farm protocol was to administer oxytetracycline to turkey poults prophylactically. This was discontinued when the farm transitioned to organic practices, probably explaining the significant reduction in the proportion of isolates resistant to the related antibiotics doxycycline and tetracycline from the organic litter (Figure 2). Gentamicin resistance has also been shown to be linked to tetracycline resistance on plasmids found in pseudomonas (Herrick 2014). The turkeys no longer received prophylactic antibiotics, therefore the enterococci excreted onto the litter had not been exposed to antibiotic residues or metabolites. Previous exposure has been linked with increasing antibiotic resistance (Schwaiger 2009; Zhang 2015).

Increases in resistance to streptomycin, erythromycin and ciprofloxacin were less marked - this finding may be due to sample size (Figure 3). It is also possible that increased resistance to the three antibiotics was random as each can be conferred by point mutations (Leavis 2006; Occhialini 1997; Traub 1968). Furthermore, it may be due to competition with antibiotic producing bacteria and fungi such as *Streptomyces*. As in the present study, in a survey comparing *Enterococcus* isolates from turkey farms, conventional farms had higher proportions of resistant isolates than newly organic farms; however, isolates from organic farms did exhibit a greater proportion of resistant isolates to some individual antibiotics (Sapkota 2011).

Individual isolates were likely to exhibit resistance to more antibiotics if they were obtained from the litter of conventionally raised turkeys (Figure 3). Resistance profiles (i.e. the individual antibiotics an isolate is resistant to) varied by rearing practice

(data not shown), which is consistent with previous comparisons of organic and conventional poultry farms (Sapkota 2011; Sapkota 2014).

This study suggests that there can be a rapid effect on levels of antibiotic resistance in enterococci when farms switch to organic practices. This is not unexpected, as the grow house was completely emptied of all litter, cleaned, and refilled with fresh wood shavings between turkey cohorts, and a new source of birds was used. Future studies should investigate multiple farms that were transitioning to organic practices to determine if the changes seen in this study were specific to the site or were common in farms rearing birds organically, and antibiotic-free. Additionally, resistance genotypes should be characterized for conventional and organic litter isolates and resistance genes should be quantified in the litter before and after the transition to organic practices.

Growth and persistence of *E. faecium* in turkey litter was affected by both temperature and moisture. Temperature was a significant factor in determining the survival time, with slower die-off at 5°C. Previous studies have also shown enteric bacteria persist longer in soil and sand at colder temperatures (Cools 2001; Howell 1996; Ishii 2006). At 30°C moisture affected length of time of survival. In moist litter *E. faecium* grew steadily for 14 days, followed by a rapid die-off during the next two weeks. It is possible that the enterococci rapidly mineralized labile, available carbon during the first two weeks due to increased microbial activity driven by the higher water content (Orchard 1983; Davidson 1998). It is also possible that available nutrients were easier to access in the higher water content (Orchard 1983). However, in dry litter the bacteria died off steadily over the course of one month. Moisture content and temperature have been shown to be the primary factors controlling the survival of fecal organisms in soil,

animal waste, and soils amended with organic wastes (Cools 2001; Desmarais 2002; Gerba 1975; Reddy 1981). Experiments using sterilized soil and sand have shown less die-off of FIB (Carpenter 2013; Hartz 2008). This is possibly due to reduced competition for resources with other microbes, as well as lack of top-down population control by bacterivores.

In all treatments *E. faecium* was still detectable after 28 days, suggesting a risk of survival in stored litter over considerable time (Figure 5). Additionally, there was a significant interaction between temperature and moisture such that litter should be land applied during warm and dry periods (Table 3; Table 4; Table 5). Zaleski (2005) showed re-growth of enterococci and other pathogens after composting. Thus, depending on storage time, untreated turkey litter applied to the land may pose a significant risk of contaminating drinking and recreational waters with FIB for up to a month or more after application.

This study has demonstrated that under certain environmental conditions litter can act as a reservoir for the fecal indicator bacterium *Enterococcus*. In some circumstances enterococci can persist or even grow for an extended period of time. Best management practices for processing used turkey litter including composting, and windrowing were important to reduce bacterial levels before amending soil with the litter. Policy makers and farmers may be able to utilize this information to determine the best season and under which conditions litter should be applied to the land.

Conclusion

This study has answered five research questions outlined earlier as follows. Were enterococci present in fresh or used litter on turkey farms? Enterococci were not present in detectable concentrations in fresh wood shavings, however, after turkeys were reared on the bedding, enterococci were detectable in high concentrations. What were common species of enterococci in the litter of organically and conventionally raised turkeys? *E. faecium* was the most prevalent species in litter from both conventionally and organically raised turkeys. The significance of differences in proportions of other species could not be assessed due to the limited numbers of these isolates. Is there a difference in the antibiotic resistance of enterococci isolated from the litter of organically and conventionally raised birds? The transition to organic rearing practices resulted in isolates resistant to fewer antibiotics (Table 3) and significant differences in the proportions of isolates resistant to 6 of the 12 antibiotics tested (Figure 2; Figure 3). Does the amount of moisture in litter affect survival and persistence of *Enterococcus*? Enterococci in moist litter were able to grow at 30°C, while all dry litter experiments resulted in steady die-off of populations over time. Does air temperature (5°C, 30°C) affect survival and persistence of *Enterococcus* in litter? Yes, long term survival was found at 5°C, with shorter survival at 30°C depending on litter moisture, i.e. survival at 30°C is higher in wet conditions than in dry. *E. faecium* was able to persist at levels above 10^5 CFU/gdw which are still quite high and pose a risk of contaminating water or crops.

Results of the microcosm experiments suggest that enterococci persist in cool environments longer than warm environments. However, the interaction between moisture and temperature play an important role in determining the decay of enterococci in litter.

Future research should include use of *E. faecium* isolates obtained from turkey litter, to determine if strain may be a factor in survival. Additionally, other species isolated from the litter, such as *E. durans* and *E. faecalis*, should be tested to assess the role that species plays in the persistence and decay of enterococci in litter. Additional studies were needed to determine if competition with other bacteria and predation affect the survival of enterococci in litter. The possibility of enterococci surviving longer than a month in litter requires additional research to include longer incubation periods with more sampling dates to improve the resolution of the survival dynamics.

Although the overall trends were the same in both experiments, there was some variability in the results of the two experiments incubated at 30˚C. This may be due to variations in the litter, such as available nutrient levels, that can result from multiple autoclave cycles, as used when preparing the medium or possibly undetected contamination that may have led to competition for resources. Additional studies should also be performed to determine if turkey rearing protocols affect the survival of enterococci. At the molecular level it will be important to determine which genes were upregulated when *E. faecium* is stressed under different environmental conditions. More farms transitioning to organic rearing practices should be sampled before and after the transition to garner a clearer understanding of how that transition affects the antibiotic resistance of enterococci in the turkey gut and in the litter.

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Appendix 1: Nutrient Analysis of Turkey Litter

PENNSTATE

(814) 863-0841 Fax: (814) 862-1540
Agricultural Analytical Services Laboratory
The Pennsylvania State University
University Park, PA 16802
www.aasl.psu.edu

Analysis Report For:	Copy To:
Steven McBride Biology Dept Bioscience Bldg , MSC 7801 Harrisonburg VA 22807	Joanna Mott

LAB ID:	SAMPLE ID:	REPORT DATE:	DATE SAMPLED:	COUNTY:	MATERIAL:	TYPE:	STORAGE SYSTEM:
M11311	Tur_Lot_01	4/6/2015			Other Treatment	Turkeys	Bedded Pack or Litter

MANURE ANALYSIS REPORT

Results on as sampled (wet weight) basis

Analyte		lb/ton	lb/1000 gal
Solids:	96.3 %		
Total Nitrogen (N)		117.53	490.11
Ammonium N (NH_4-N)		5.03	20.96
Calculated Organic N		112.51	469.16
Total Phosphate (P_2O_5)		76.29	318.12
Total Potash (K_2O)		58.27	242.99

Optional Test Results:	pH	Carbon (C) (%)	C:N Ratio	Ash (%)	Volatiles (%)	Nitrate Nitrogen (lb/ton)	Nitrate Nitrogen (lb/1000 gal)	Soluble Salts mmhos/cm	Soluble Salts manure:water	PSC*

*P Source Coefficient for use in Pennsylvania P Index

Comments:

- The enclosed fact sheet "Using Your Manure Analysis Report" provides information to help you interpret this report and calculate appropriate manure application rates for your crops.
- Manure nutrients are not all equivalent to fertilizer nutrients. Phosphorus and potassium can be substituted directly for fertilizer to meet your soil test recommendation. Nitrogen (N) availability varies with handling. This must be accounted for in utilizing manure to meet soil test N recommendations. See the enclosed fact sheet "Using Your Manure Analysis Report"

Nutrient contents are presented as both "lb/ton" and "lb/1000 gal". Choose results with the units that are most convenient for you. An assumed manure density of 8.34 lbs per gal was used to calculate results on a lb/1000 gal basis.

PENNSTATE

1855

(814) 863-0841 Fax. (814) 863-4540
Agricultural Analytical Services Laboratory
The Pennsylvania State University
University Park, PA 16802
www.aasl.psu.edu

Analysis Report For:	Copy To:
Steven McBride Biology Dept Bioscience Bldg , MSC 7801 Harrisonburg VA 22807	Joanna Mott

LAB ID:	SAMPLE ID:	REPORT DATE:	DATE SAMPLED:	COUNTY:	MATERIAL:	TYPE:	STORAGE SYSTEM:
M11310	Tur_Lot_02	4/6/2015			Other Treatment	Turkeys	Bedded Pack or Litter

MANURE ANALYSIS REPORT

Results on as sampled (wet weight) basis

Analyte	lb/ton	lb/1000 gal
Solids: 96.4 %		
Total Nitrogen (N)	94.55	394.29
Ammonium N (NH_4-N)	5.80	24.19
Calculated Organic N	88.75	370.10
Total Phosphate (P_2O_5)	83.91	349.89
Total Potash (K_2O)	74.97	312.62

Optional Test Results:	pH	Carbon (C) (%)	C:N Ratio	Ash (%)	Volatiles (%)	Nitrate Nitrogen (lb/ton)	Nitrate Nitrogen (lb/1000 gal)	Soluble Salts mmhos/cm	Soluble Salts manure:water	PSC*

*P Source Coefficient for use in Pennsylvania P Index

Comments:

- The enclosed fact sheet "Using Your Manure Analysis Report" provides information to help you interpret this report and calculate appropriate manure application rates for your crops.
- Manure nutrients are not all equivalent to fertilizer nutrients. Phosphorus and potassium can be substituted directly for fertilizer to meet your soil test recommendation. Nitrogen (N) availability varies with handling. This must be accounted for in utilizing manure to meet soil test N recommendations. See the enclosed fact sheet "Using Your Manure Analysis Report"

Nutrient contents are presented as both "lb/ton" and "lb/1000 gal". Choose results with the units that are most convenient for you. An assumed manure density of 8.34 lbs per gal was used to calculate results on a lb/1000 gal basis.

Appendix 2: Supplementary Data

Table A2A: Means and standard deviations of combined experiments

log10(CFU/gdw)					
Temperature	Moisture	Days incubated	Obs	Mean	Std Dev
5	dry	0	6	7.3627538	0.0931117
		1	6	7.5062380	0.2648951
		7	6	7.3983153	0.1217711
		14	6	7.2108631	0.1816043
		28	6	7.2128926	0.2165498
	wet	0	6	7.6058341	0.1156968
		1	6	7.7226286	0.2079088
		7	6	7.5245186	0.1287667
		14	6	7.3241223	0.1038818
		28	6	7.3592520	0.1232340
30	dry	0	6	7.1183452	0.2574320
		1	6	6.8376028	0.5483408
		7	6	6.1088982	0.9246770
		14	6	5.4977245	1.5655358
		28	5	5.4283167	1.1609422
	wet	0	6	7.6166698	0.0475249
		1	6	7.8812680	0.8285410
		7	4	8.4894668	0.3251655
		14	5	8.8579016	0.2534215
		28	5	7.7462145	0.5312883

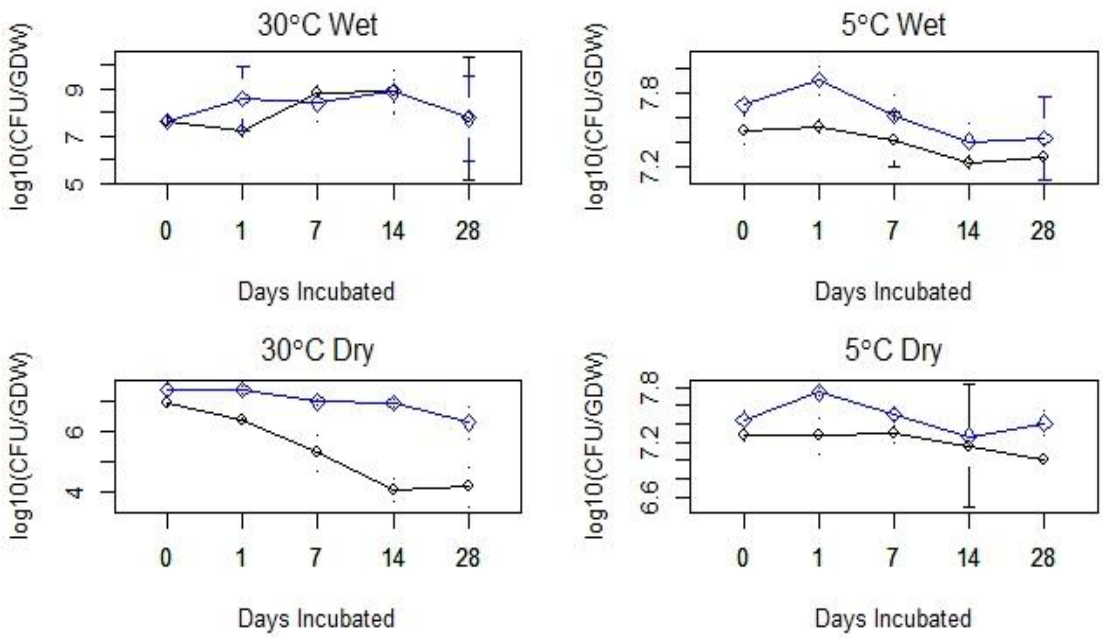

Figure: A2A: Comparison of duplicate runs of each experiment. Bars represent standard error.

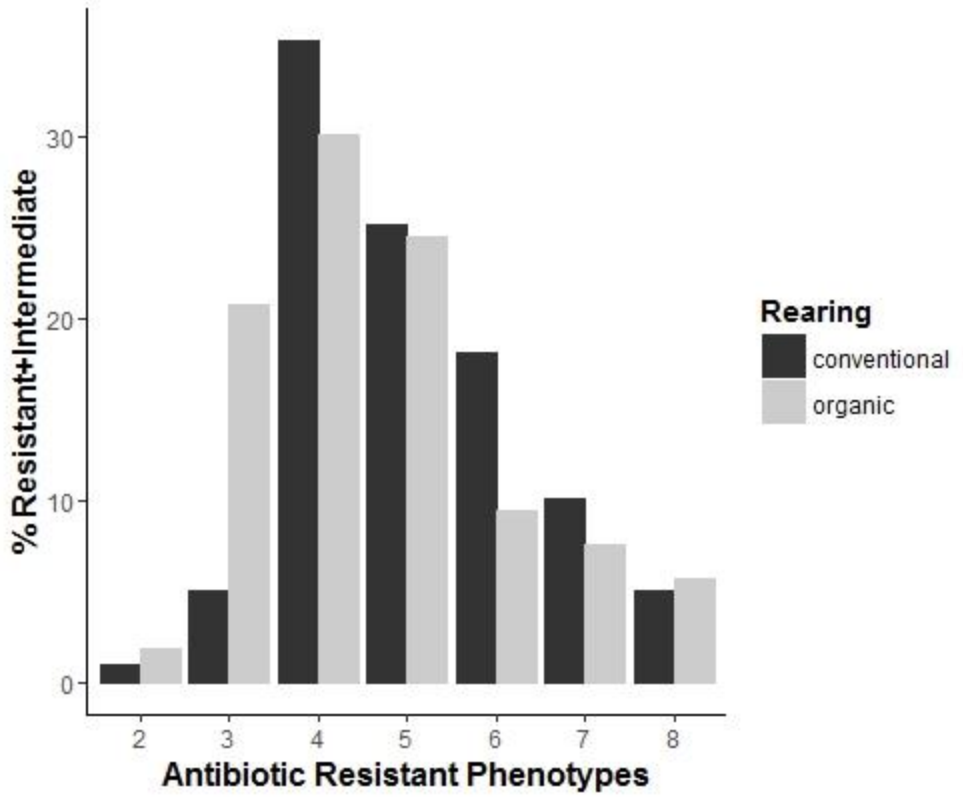

Figure: A2B: Isolates exhibiting resistance+intermediate phenotypes to one or more antibiotics (W=3116; p=0.0499).

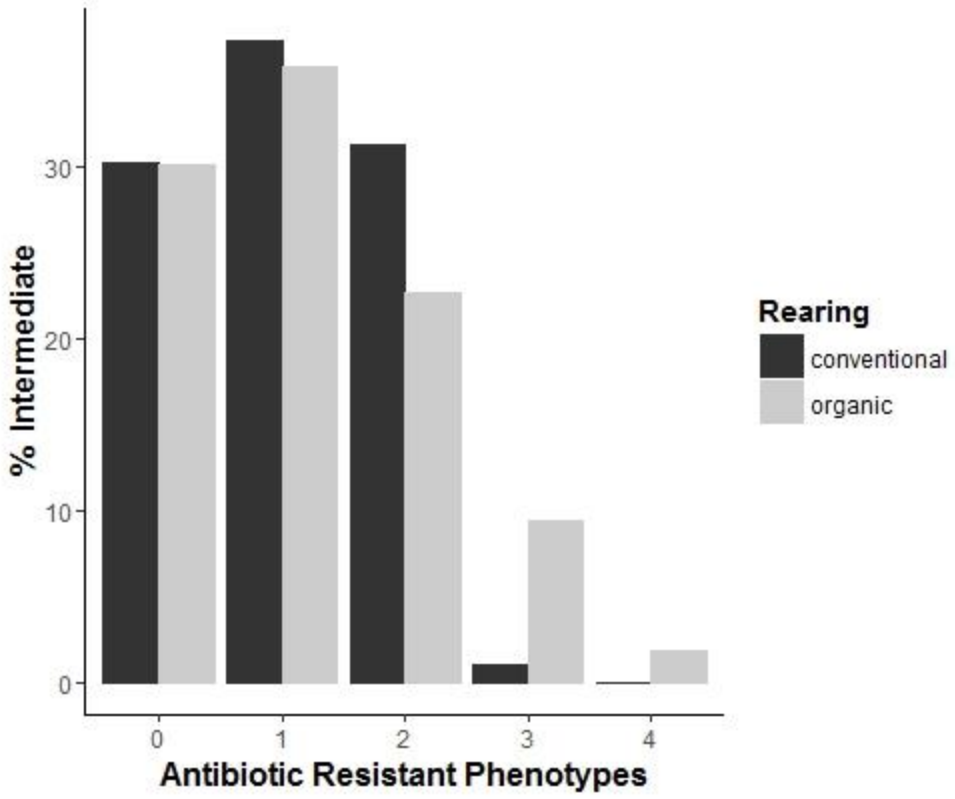

Figure A2C: Isolates exhibiting intermediate phenotypes to one or more antibiotics (W=2504; p=0.6276).

Table A2B: Proportions of isolates with resistant or intermediate phenotypes.

Antibiotic	Phenotype	Conventional	Organic
Ampicillin	Resistant	0.10	0.13
	Intermediate	0.00	0.00
Ciprofloxacin	Resistant	0.34	0.60
	Intermediate	0.55	0.31
Chloramphenicol	Resistant	0.00	0.00
	Intermediate	0.00	0.00
Doxycycline	Resistant	0.67	0.44
	Intermediate	0.27	0.13
Erythroycin	Resistant	0.43	0.52
	Intermediate	0.04	0.23
Gentamicin	Resistant	0.73	0.27
	Intermediate	0.05	0.17
Linezolid	Resistant	0.00	0.00
	Intermediate	0.00	0.00
Quinipristin/ Dalfopristin	Resistant	0.24	0.10
	Intermediate	0.00	0.13
Rifampin	Resistant	0.03	0.00
	Intermediate	0.06	0.08
Streptomycin	Resistant	0.51	0.87
	Intermediate	0.05	0.12
Tetracycline	Resistant	0.98	0.58
	Intermediate	0.00	0.00
Vancomycin	Resistant	0.00	0.00
	Intermediate	0.02	0.00

Appendix 3: SAS Code for Linear Mixed effects model

```
data survival;
 input Rep $ Temperature  Moisture $ Experiment $ days_incubated Tube_ID $ Per_H20 DryWeight
Calculation log10;
datalines;
A 30 dry 30D_A 0 ADM-A 0.090285578 14.61911076 7890926.828
6.897128016
A 30 dry 30D_A 0 ADM-B 0.090285578 14.53723647 7789765.746
6.891524398
A 30 dry 30D_A 0 ADM-C 0.090285578 14.52813932 7503254.953
6.875249704
A 30 dry 30D_A 1 ADM-A 0.090285578 14.50994503 2042277.436
6.310114739
A 30 dry 30D_A 1 ADM-B 0.090285578 14.55543076 2108605.866
6.32399541
A 30 dry 30D_A 1 ADM-C 0.090285578 14.55543076 2399448.054
6.380111352
A 30 dry 30D_A 7 ADM-A 0.090285578 14.58272219 312070.2208
5.494252328
A 30 dry 30D_A 7 ADM-B 0.090285578 14.58272219 108861.7049
5.036875132
A 30 dry 30D_A 7 ADM-C 0.090285578 14.4826536 197305.0022
5.295138096
A 30 dry 30D_A 14 ADM-A 0.090285578 14.49175075 15336.31125
4.185720914
A 30 dry 30D_A 14 ADM-B 0.090285578 14.54633361 12877.81547
4.109842198
A 30 dry 30D_A 14 ADM-C 0.090285578 14.58272219 8273.489575
3.917688724
A 30 dry 30D_A 28 ADM-B 0.090285578 14.55543076 13087.89848
4.116869917
A 30 dry 30D_A 28 ADM-C 0.090285578 14.57362504 16557.3081
4.21898973
B 30 dry 30D_B 0 ADM-A 0.098614399 14.36808648 19887825.73
7.298587306
B 30 dry 30D_B 0 ADM-B 0.098614399 14.55737746 20283529.84
7.307143535
B 30 dry 30D_B 0 ADM-C 0.098614399 14.39512805 27570091.68
7.44043821
B 30 dry 30D_B 1 ADM-A 0.098614399 14.38611419 20525000.43
7.312283175
B 30 dry 30D_B 1 ADM-B 0.098614399 14.38611419 24276882.23
7.385192911
B 30 dry 30D_B 1 ADM-C 0.098614399 14.33203106 20602453.26
7.313918938
B 30 dry 30D_B 7 ADM-A 0.098614399 14.4672389 9063754.385
6.957308128
B 30 dry 30D_B 7 ADM-B 0.098614399 14.4672389 9173485.068
6.962534358
B 30 dry 30D_B 7 ADM-C 0.098614399 14.38611419 8077580.814
6.907281312
B 30 dry 30D_B 14 ADM-A 0.098614399 14.52132203 7433896.153
6.87121649
```

B	30	dry	30D_B	14	ADM-B	0.098614399	14.41315576	9178535.836
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B	30	dry	30D_B	14	ADM-C	0.098614399	14.36808648	8691716.43
6.939105549								
B	30	dry	30D_B	28	ADM-A	0.098614399	14.42216962	1056706.474
6.023954368								
B	30	dry	30D_B	28	ADM-B	0.098614399	14.33203106	2584537.148
6.412382779								
B	30	dry	30D_B	28	ADM-C	0.098614399	14.4672389	2340921.229
6.3693868								
B	5	wet	5W_B	0	AWM-A	0.36306292	10.17825453	
4.77E+07	7.678761853							
B	5	wet	5W_B	0	AWM-B	0.36306292	10.23557887	5.21E+07
7.716940602								
B	5	wet	5W_B	0	AWM-C	0.36306292	10.15914642	5.31E+07
7.725335431								
B	5	wet	5W_B	1	AWM-A	0.36306292	10.2292095	
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B	5	wet	5W_B	14	AWM-B	0.36306292	10.17825453	2.84E+07
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B	5	wet	5W_B	14	AWM-C	0.36306292	10.17188516	2.22E+07
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B	5	wet	5W_B	28	AWM-A	0.36306292	10.21647076	
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B	5	wet	5W_B	28	AWM-B	0.36306292	10.17825453	1.90E+07
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A	5	wet	5W_A	0	AWM-B	0.365046669	10.15290376	30020968.12
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A	5	wet	5W_A	0	AWM-C	0.365046669	10.12750562	30096255.82
7.47851247								
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7.550410188								
A	5	wet	5W_A	1	AWM-C	0.365046669	10.09575796	32392317.78
7.510442024								
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31056234.8	7.492148802							
A	5	wet	5W_A	7	AWM-B	0.365046669	10.12115609	29174038.75
7.464996556								

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A	5	wet	5W_A	14	AWM-B	0.365046669	10.15925329	16876240.32
7.227275701								
A	5	wet	5W_A	14	AWM-C	0.365046669	10.12115609	16626065.1
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7.249672416								
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B	30	wet	30W_B	1	AWM-A	0.372732958	10.11781739	
577397256.5	8.761474716							
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8.992356279								
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B	30	wet	30W_B	7	AWM-A	0.372732958	10.02372733	
190049064.3	8.278865736							
B	30	wet	30W_B	7	AWM-B	0.372732958	10.02372733	544807317.7
8.736242932								
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8.146346813								
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284539417.4	8.454142438							
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B	30	wet	30W_B	28	AWM-B	0.372732958	10.01745466	12994817.99
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B	30	wet	30W_B	28	AWM-C	0.372732958	10.05509068	39154296.31
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B	5	dry	5D_B	0	ADM-A	0.089663761	14.58358655	3.07E+07
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B	5	dry	5D_B	0	ADM-B	0.089663761	14.58358655	2.66E+07
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B	5	dry	5D_B	0	ADM-C	0.089663761	14.62910336	2.63E+07
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B	5	dry	5D_B	1	ADM-A	0.089663761	14.56537983	5.62E+07
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B	5	dry	5D_B	7	ADM-A	0.089663761	14.48344956	3.00E+07	7.477592285
B	5	dry	5D_B	7	ADM-B	0.089663761	14.48344956	3.38E+07	7.528392439
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B	5	dry	5D_B	14	ADM-B	0.089663761	14.65641345	1.67E+07	7.222206747
B	5	dry	5D_B	14	ADM-C	0.089663761	14.53806974	1.97E+07	7.293479491
B	5	dry	5D_B	28	ADM-A	0.089663761	14.50165629	2.26E+07	7.353163347
B	5	dry	5D_B	28	ADM-B	0.089663761	14.51075965	2.84E+07	7.453996933
B	5	dry	5D_B	28	ADM-C	0.089663761	14.59268991	2.61E+07	7.416789622
A	5	dry	5D_A	0	ADMA	0.093707278	14.52787233	1.92E+07	7.284024387
A	5	dry	5D_A	0	ADMB	0.093707278	14.4191172	2.03E+07	7.306592885
A	5	dry	5D_A	0	ADMC	0.093707278	14.46443184	1.80E+07	7.255256203
A	5	dry	5D_A	1	ADMA	0.093707278	14.47349476	2.06E+07	7.314298175
A	5	dry	5D_A	1	ADMB	0.093707278	14.46443184	1.51E+07	7.180291441
A	5	dry	5D_A	1	ADMC	0.093707278	14.41005427	2.05E+07	7.311561062
A	5	dry	5D_A	7	ADMA	0.093707278	14.43724306	2.00E+07	7.301300854
A	5	dry	5D_A	7	ADMB	0.093707278	14.43724306	2.09E+07	7.319983067
A	5	dry	5D_A	7	ADMC	0.093707278	14.48255769	1.78E+07	7.249383481
A	5	dry	5D_A	14	ADMA	0.093707278	14.46443184	2.92E+07	7.465293991
A	5	dry	5D_A	14	ADMB	0.093707278	14.47349476	1.05E+07	7.022411559
A	5	dry	5D_A	14	ADMC	0.093707278	14.48255769	9.57E+06	6.981048503
A	5	dry	5D_A	28	ADMA	0.093707278	14.41005427	9.91E+06	6.996290627
A	5	dry	5D_A	28	ADMB	0.093707278	14.41005427	1.04E+07	7.018245203
A	5	dry	5D_A	28	ADMC	0.093707278	14.4191172	1.09E+07	7.038870071
A	30	wet	30W_A	0	AWM-A	0.374	10.0473	4.39E+07	7.64270916
A	30	wet	30W_A	0	AWM-B	0.374	10.03478	4.84E+07	7.684927305
A	30	wet	30W_A	0	AWM-C	0.374	9.9534	3.60E+07	7.556850716
A	30	wet	30W_A	1	AWM-A	0.374	10.0473	1.23E+07	7.090758967
A	30	wet	30W_A	1	AWM-B	0.374	9.9847	1.97E+07	7.294800399

```
A 30 wet 30W_A 1 AWM-C0.374 9.99096 1.62E+07 7.209706686
A 30 wet 30W_A 7 AWM-A 0.374 9.97844 6.26E+08
8.796411836
A 30 wet 30W_A 14 AWM-B0.374 10.02852 7.91E+08
8.898446893
A 30 wet 30W_A 14 AWM-C0.374 10.00348 6.67E+08
8.823811916
A 30 wet 30W_A 28 AWM-B0.374 9.99722 8.89E+07 7.949022512
A 30 wet 30W_A 28 AWM-C0.374 10.05982 3.47E+07
7.540546205
;
run;
ods graphics off;
proc print data = survival;
run;
symbol1 value = diamond color = red;
symbol2 value = star color = black;
proc gplot data = survival;
  title1 'Temperature = 5 and Moisture = Dry';
  where temperature = '5' and moisture = 'dry';
  plot log10 * days_incubated = rep;
run;
proc gplot data = survival;
  title1 'Temperature = 30 and Moisture = Dry';
  where temperature = '30' and moisture = 'dry';
  plot log10 * days_incubated = rep;
run;
proc gplot data = survival;
  title1 'Temperature = 5 and Moisture = Wet';
  where temperature = '5' and moisture = 'wet';
  plot log10 * days_incubated = rep;
run;
proc gplot data = survival;
  title1 'Temperature = 30 and Moisture = Wet';
  where temperature = '30' and moisture = 'wet';
  plot log10 * days_incubated = rep;
run;
proc means data = survival mean std;
 var log10;
 class Temperature Moisture days_incubated;
run;
*  Model 1:
    model statement specifies temperature, moisture, days_incubated and all possible interactions
     (2 and 3-way) as fixed effects
    random statement specifies Rep(temperature*moisture), Rep*Days_Incubated(Temperature*Moisture)
     as random effects - model also includes a residual random error by default

    outpred = PRED_SURVIVAL residual tells SAS to save the estimated residuals for checking of
              assumptions of normality and homogenenous variances.

    ddfm = kr specifies the degrees of freedom method to use for hypthesis tests

    AIC = model fit for this model = 57;
/*
proc mixed data = survival;
 class temperature moisture rep days_incubated;
```

```
 model log10 = temperature moisture days_incubated
            temperature*moisture temperature*days_incubated moisture*days_incubated
            temperature*moisture*days_incubated / outpred = PRED_SURVIVAL1 solution residual ddfm =
kr;
 random rep(temperature*moisture)
       rep*days_incubated(temperature*moisture);
 estimate 'moisture x temp interaction test at Day 0'
        temperature     0  0
        moisture        0  0
                        days_incubated  0 0 0 0 0
                        temperature*moisture    1 -1
                                        -1  1
                        temperature*days_incubated  0 0 0 0 0
                                        0 0 0 0 0
                        moisture*days_incubated     0 0 0 0 0
                                        0 0 0 0 0
                        temperature*moisture*days_incubated   1 0 0 0 0
                                        -1 0 0 0 0

          -1 0 0 0 0

1 0 0 0 0;
run;

*  The following proc univariate and proc gplot check on normality of homogeneity
    of variance for the residuals - there is some evidence that variance of residuals
    changes with temperature*moisture combination;
proc univariate data = PRED_SURVIVAL1 noprint;
  var StudentResid;
  histogram;
  qqplot;
run;
SYMBOL1 value = circle;
SYMBOL2;
TITLE1;
proc gplot data = PRED_SURVIVAL1;
  plot StudentResid * Pred;
  plot StudentResid * Moisture;
  plot StudentResid * Temperature;
  plot StudentResid * days_incubated;
run;
*/;
* Model 2 was fit due to evidence of heterogeneous error variances in residual plots
 from Model 1  -- repeated / group = temperature*moisture fits a separate residual
 variance for each temperature*moisture combination
 Better model fit with AIC = -6.3, thus tentatively use this model for inference, testing, etc
 Histogram, QQ-Plot look better for normality
 No more evidence of heterogeneous error variation
 There is evidence of 3-way interaction, which means evidence that two-way interaction
 exist and depends on levels of 3rd factor.  For example, there is evidence
 that moisture and days incubated interact and this interaction depends on levels of
 temperature.  Might look at some contrasts comparing
 wet and dry at each day and then do this for each temperature;
proc mixed data = survival;
 class temperature moisture rep days_incubated;
 model log10 = temperature moisture days_incubated
```

```
            temperature*moisture temperature*days_incubated moisture*days_incubated
            temperature*moisture*days_incubated / outpred = PRED_SURVIVAL2 solution residual ddfm =
kr;
  random rep(temperature*moisture)
        rep*days_incubated(temperature*moisture);
  repeated / group = temperature*moisture;
run;
proc univariate data = PRED_SURVIVAL2 noprint;
   var StudentResid;
   histogram;
   qqplot;
run;
SYMBOL1 value = circle;
SYMBOL2;
TITLE1;
proc gplot data = PRED_SURVIVAL2;
   plot StudentResid * Pred;
   plot StudentResid * Moisture;
   plot StudentResid * Temperature;
   plot StudentResid * days_incubated;
run;
*ods pdf close;
 * Model 3:  Same as Model 2, but written in equivalent form to
          facilitate the writing of estimate statements to make
          various comparisons at each Day;
proc mixed data = survival;
  class temperature moisture rep days_incubated;
  model log10 = temperature*moisture*days_incubated / noint solution  ddfm = kr;
  random rep(temperature*moisture)
        rep*days_incubated(temperature*moisture);
  repeated / group = temperature*moisture;
* In all of the estimate statements below the sequence of 20 digits
  correspond to the 20 combinations of temperature, moisture, and day:
  1st set of 5 correspond to temp = 5, moisture = dry, five days(0,1,7,14,28)
  2nd set of 5 correspond to temp = 5, moisture = wet, five days(0,1,7,14,28)
  3rd set of 5 correspond to temp = 10, moisture=dry, five days(0,1,7,14,28)
  4th set of 5 correspond to temp = 10, moisture=wet, five days(0,1,7,14,28);
* The estimate statements below are appropriate for Day = 0 comparisons;
* The one estimate statement below is for testing the two way interaction
  between temperature and moisture at Day 0;
  estimate 'interaction between moisture and temperature at Day 0'
          temperature*moisture*days_incubated
                        1 0 0 0 0   -1 0 0 0 0   -1 0 0 0 0   1 0 0 0 0;
* The two estimate statements below are appropriate if the interaction
  between moisture and temperature at Day 0 IS NOT significant, then
  compare temperatures (averaged over moisture) and compare
  compare moistures (averaged over temperature);
  estimate 'compare temperatures 5 versus 30 (averaged over moistures) at Day 0'
          temperature*moisture*days_incubated
           .5 0 0 0 0   .5 0 0 0 0   -.5 0 0 0 0   -.5 0 0 0 0;
  estimate 'compare moistures dry versus wet (averaged over temperature) at Day 0'
          temperature*moisture*days_incubated
           .5 0 0 0 0   -.5 0 0 0 0   .5 0 0 0 0   -.5 0 0 0 0;
* The four estimate statements below are appropriate if the two-way
  interaction between moisture and temperature at Day 0 IS significant.
  Then compare temperatures at each level of moisture and
```

```
            compare moistures at each level of temperature;
  estimate 'compare temp of 5 versus temp of 30 at moisture=dry, Day = 0'
        temperature*moisture*days_incubated
                    1 0 0 0 0   0 0 0 0 0   -1 0 0 0 0   0 0 0 0 0;
  estimate 'compare temp of 5 versus temp of 30 at moisture=WET and Day = 0'
        temperature*moisture*days_incubated
                    0 0 0 0 0   1 0 0 0 0   0 0 0 0 0   -1 0 0 0 0;
  estimate 'compare moisture of dry versus wet at temp = 5, Day = 0'
        temperature*moisture*days_incubated
         1 0 0 0 0  -1 0 0 0 0   0 0 0 0 0   0 0 0 0 0;
  estimate 'compare moisture of dry versus wet at temp = 30, Day = 0'
        temperature*moisture*days_incubated
         0 0 0 0 0   0 0 0 0 0   1 0 0 0 0  -1 0 0 0 0;
* Additional estimate statements can be written for comparisons of temperature
  and moisture at each of Days 1, 7, 14, 28;
* The one estimate statement below is for testing the two way interaction
  between temperature and moisture at Day 0;
  estimate 'interaction between moisture and temperature at Day 1'
        temperature*moisture*days_incubated
                    0 1 0 0 0   0 -1 0 0 0   0 -1 0 0 0   0 1 0 0 0;
                          * The two estimate statements below are appropriate if the interaction
  between moisture and temperature at Day 1 IS NOT significant, then
  compare temperatures (averaged over moisture) and compare
  compare moistures (averaged over temperature);
  estimate 'compare temperatures 5 versus 30 (averaged over moistures) at Day 1'
        temperature*moisture*days_incubated
         0 .5 0 0 0   0 .5 0 0 0  0 -.5 0 0 0  0 -.5 0 0 0;
  estimate 'compare moistures dry versus wet (averaged over temperature) at Day 1'
        temperature*moisture*days_incubated
         0 .5 0 0 0  0 -.5 0 0 0  0 .5 0 0 0   0 -.5 0 0 0;
  estimate 'interaction between moisture and temperature at Day 7'
        temperature*moisture*days_incubated
                    0 0 1 0 0   0 0 -1 0 0   0 0 -1 0 0   0 0 1 0 0;

                                          * The two estimate statements below are appropriate if the
interaction
  between moisture and temperature at Day 7 IS NOT significant, then
  compare temperatures (averaged over moisture) and compare
  compare moistures (averaged over temperature);
  estimate 'compare temperatures 5 versus 30 (averaged over moistures) at Day 7'
        temperature*moisture*days_incubated
         0 0 .5 0 0   0 0 .5 0 0  0 0 -.5 0 0  0 0 -.5 0 0;
  estimate 'compare moistures dry versus wet (averaged over temperature) at Day 7'
        temperature*moisture*days_incubated
         0 0 .5 0 0  0 0 -.5 0 0  0 0 .5 0 0   0 0 -.5 0 0;
  estimate 'interaction between moisture and temperature at Day 14'
        temperature*moisture*days_incubated
                    0 0 0 1 0   0 0 0 -1 0   0 0 0 -1 0   0 0 0 1 0;
* The four estimate statements below are appropriate if the two-way
  interaction between moisture and temperature at Day 14 IS significant.
  Then compare temperatures at each level of moisture and
            compare moistures at each level of temperature;
  estimate 'compare temp of 5 versus temp of 30 at moisture=dry, Day = 14'
        temperature*moisture*days_incubated
                    0 0 0 1 0   0 0 0 0 0   0 0 0 -1 0   0 0 0 0 0;
  estimate 'compare temp of 5 versus temp of 30 at moisture=WET and Day = 14'
```

```
        temperature*moisture*days_incubated
                        0 0 0 0 0   0 0 0 1 0   0 0 0 0 0   0 0 0 -1 0;
estimate 'compare moisture of dry versus wet at temp = 5, Day = 14'
        temperature*moisture*days_incubated
         0 0 0 1 0   0 0 0 -1 0   0 0 0 0 0   0 0 0 0 0;
estimate 'compare moisture of dry versus wet at temp = 30, Day = 14'
        temperature*moisture*days_incubated
         0 0 0 0 0   0 0 0 0 0   0 0 0 1 0   0 0 0 -1 0;
estimate 'interaction between moisture and temperature at Day 28'
        temperature*moisture*days_incubated
                         0 0 0 0 1   0 0 0 0 -1   0 0 0 0 -1   0 0 0 0 1;
                                * The four estimate statements below are appropriate if the two-way
interaction between moisture and temperature at Day 28 IS significant.
Then compare temperatures at each level of moisture and
              compare moistures at each level of temperature;
estimate 'compare temp of 5 versus temp of 30 at moisture=dry, Day = 28'
        temperature*moisture*days_incubated
                        0 0 0 0 1   0 0 0 0 0   0 0 0 0 -1   0 0 0 0 0;
estimate 'compare temp of 5 versus temp of 30 at moisture=WET and Day = 28'
        temperature*moisture*days_incubated
                        0 0 0 0 0   0 0 0 0 1   0 0 0 0 0   0 0 0 0 -1;
estimate 'compare moisture of dry versus wet at temp = 5, Day = 28'
        temperature*moisture*days_incubated
         0 0 0 0 1   0 0 0 0 -1   0 0 0 0 0   0 0 0 0 0;
estimate 'compare moisture of dry versus wet at temp = 30, Day = 28'
        temperature*moisture*days_incubated
         0 0 0 0 0   0 0 0 0 0   0 0 0 0 1   0 0 0 0 -1;
           run;
```

Appendix 4: SAS output for linear mixed effects model

Table A4A: Raw data for survival experiments

Obs	Rep	Temperature	Moisture	Experiment	days_incubated	Tube_ID	Per_H20	DryWeight	Calculation	log10
1	A	30	Dry	30D_A	0	ADM-A	9.03%	14.62	7.89E+06	6.90
2	A	30	Dry	30D_A	0	ADM-B	9.03%	14.54	7.79E+06	6.89
3	A	30	Dry	30D_A	0	ADM-C	9.03%	14.53	7.50E+06	6.88
4	A	30	dry	30D_A	1	ADM-A	9.03%	14.51	2.04E+06	6.31
5	A	30	dry	30D_A	1	ADM-B	9.03%	14.56	2.11E+06	6.32
6	A	30	dry	30D_A	1	ADM-C	9.03%	14.56	2.40E+06	6.38
7	A	30	dry	30D_A	7	ADM-A	9.03%	14.58	3.12E+05	5.49
8	A	30	dry	30D_A	7	ADM-B	9.03%	14.58	1.09E+05	5.04
9	A	30	dry	30D_A	7	ADM-C	9.03%	14.48	1.97E+05	5.30
10	A	30	dry	30D_A	14	ADM-A	9.03%	14.49	1.53E+04	4.19
11	A	30	dry	30D_A	14	ADM-B	9.03%	14.55	1.29E+04	4.11
12	A	30	dry	30D_A	14	ADM-C	9.03%	14.58	8.27E+03	3.92
13	A	30	dry	30D_A	28	ADM-B	9.03%	14.56	1.31E+04	4.12
14	A	30	dry	30D_A	28	ADM-C	9.03%	14.57	1.66E+04	4.22

15	B	30	dry	30D_B	0	ADM-A	9.86%	14.37	1.99E+07	7.30
16	B	30	dry	30D_B	0	ADM-B	9.86%	14.56	2.03E+07	7.31
17	B	30	dry	30D_B	0	ADM-C	9.86%	14.40	2.76E+07	7.44
18	B	30	dry	30D_B	1	ADM-A	9.86%	14.39	2.05E+07	7.31
19	B	30	dry	30D_B	1	ADM-B	9.86%	14.39	2.43E+07	7.39
20	B	30	dry	30D_B	1	ADM-C	9.86%	14.33	2.06E+07	7.31
21	B	30	dry	30D_B	7	ADM-A	9.86%	14.47	9.06E+06	6.96
22	B	30	dry	30D_B	7	ADM-B	9.86%	14.47	9.17E+06	6.96
23	B	30	dry	30D_B	7	ADM-C	9.86%	14.39	8.08E+06	6.91
24	B	30	dry	30D_B	14	ADM-A	9.86%	14.52	7.43E+06	6.87
25	B	30	dry	30D_B	14	ADM-B	9.86%	14.41	9.18E+06	6.96
26	B	30	dry	30D_B	14	ADM-C	9.86%	14.37	8.69E+06	6.94
27	B	30	dry	30D_B	28	ADM-A	9.86%	14.42	1.06E+06	6.02
28	B	30	dry	30D_B	28	ADM-B	9.86%	14.33	2.58E+06	6.41
29	B	30	dry	30D_B	28	ADM-C	9.86%	14.47	2.34E+06	6.37
30	B	5	wet	5W_B	0	AWM-A	36.31%	10.18	4.77E+07	7.68
31	B	5	wet	5W_B	0	AWM-B	36.31%	10.24	5.21E+07	7.72
32	B	5	wet	5W_B	0	AWM-C	36.31%	10.16	5.31E+07	7.73

33	B	5	wet	5W_B	1	AWM-A	36.31%	10.23	7.82E+07	7.89
34	B	5	wet	5W_B	1	AWM-B	36.31%	10.32	7.51E+07	7.88
35	B	5	wet	5W_B	1	AWM-C	36.31%	10.23	9.16E+07	7.96
36	B	5	wet	5W_B	7	AWM-A	36.31%	10.20	4.67E+07	7.67
37	B	5	wet	5W_B	7	AWM-B	36.31%	10.20	3.53E+07	7.55
38	B	5	wet	5W_B	7	AWM-C	36.31%	10.17	4.47E+07	7.65
39	B	5	wet	5W_B	14	AWM-A	36.31%	10.21	2.74E+07	7.44
40	B	5	wet	5W_B	14	AWM-B	36.31%	10.18	2.84E+07	7.45
41	B	5	wet	5W_B	14	AWM-C	36.31%	10.17	2.22E+07	7.35
42	B	5	wet	5W_B	28	AWM-A	36.31%	10.22	3.11E+07	7.49
43	B	5	wet	5W_B	28	AWM-B	36.31%	10.18	1.90E+07	7.28
44	B	5	wet	5W_B	28	AWM-C	36.31%	10.18	3.43E+07	7.54
45	A	5	wet	5W_A	0	AWM-A	36.51%	10.10	3.61E+07	7.56
46	A	5	wet	5W_A	0	AWM-B	36.51%	10.15	3.00E+07	7.48
47	A	5	wet	5W_A	0	AWM-C	36.51%	10.13	3.01E+07	7.48
48	A	5	wet	5W_A	1	AWM-A	36.51%	10.15	3.50E+07	7.54
49	A	5	wet	5W_A	1	AWM-B	36.51%	10.10	3.55E+07	7.55
50	A	5	wet	5W_A	1	AWM-C	36.51%	10.10	3.24E+07	7.51

51	A	5	wet	5W_A	7	AWM-A	36.51%	10.12	3.11E+07	7.49
52	A	5	wet	5W_A	7	AWM-B	36.51%	10.12	2.92E+07	7.47
53	A	5	wet	5W_A	7	AWM-C	36.51%	10.13	2.10E+07	7.32
54	A	5	wet	5W_A	14	AWM-A	36.51%	10.10	1.82E+07	7.26
55	A	5	wet	5W_A	14	AWM-B	36.51%	10.16	1.69E+07	7.23
56	A	5	wet	5W_A	14	AWM-C	36.51%	10.12	1.66E+07	7.22
57	A	5	wet	5W_A	28	AWM-A	36.51%	10.22	1.86E+07	7.27
58	A	5	wet	5W_A	28	AWM-B	36.51%	10.18	1.78E+07	7.25
59	A	5	wet	5W_A	28	AWM-C	36.51%	10.13	2.13E+07	7.33
60	B	30	wet	30W_B	0	AWM-A	37.27%	10.05	3.89E+07	7.59
61	B	30	wet	30W_B	0	AWM-B	37.27%	10.00	3.84E+07	7.58
62	B	30	wet	30W_B	0	AWM-C	37.27%	10.00	4.38E+07	7.64
63	B	30	wet	30W_B	1	AWM-A	37.27%	10.12	5.77E+08	8.76
64	B	30	wet	30W_B	1	AWM-B	37.27%	10.02	9.83E+08	8.99
65	B	30	wet	30W_B	1	AWM-C	37.27%	9.99	8.68E+07	7.94
66	B	30	wet	30W_B	7	AWM-A	37.27%	10.02	1.90E+08	8.28
67	B	30	wet	30W_B	7	AWM-B	37.27%	10.02	5.45E+08	8.74
68	B	30	wet	30W_B	7	AWM-C	37.27%	9.97	1.40E+08	8.15

69	B	30	wet	30W_B	14	AWM-A	37.27%	10.04	2.85E+08	8.45
70	B	30	wet	30W_B	14	AWM-B	37.27%	10.02	1.36E+09	9.13
71	B	30	wet	30W_B	14	AWM-C	37.27%	10.00	9.52E+08	8.98
72	B	30	wet	30W_B	28	AWM-A	37.27%	10.00	3.43E+08	8.53
73	B	30	wet	30W_B	28	AWM-B	37.27%	10.02	1.30E+07	7.11
74	B	30	wet	30W_B	28	AWM-C	37.27%	10.06	3.92E+07	7.59
75	B	5	dry	5D_B	0	ADM-A	8.97%	14.58	3.07E+07	7.49
76	B	5	dry	5D_B	0	ADM-B	8.97%	14.58	2.66E+07	7.42
77	B	5	dry	5D_B	0	ADM-C	8.97%	14.63	2.63E+07	7.42
78	B	5	dry	5D_B	1	ADM-A	8.97%	14.57	5.62E+07	7.75
79	B	5	dry	5D_B	1	ADM-B	8.97%	14.63	5.30E+07	7.72
80	B	5	dry	5D_B	1	ADM-C	8.97%	14.66	5.72E+07	7.76
81	B	5	dry	5D_B	7	ADM-A	8.97%	14.48	3.00E+07	7.48
82	B	5	dry	5D_B	7	ADM-B	8.97%	14.48	3.38E+07	7.53
83	B	5	dry	5D_B	7	ADM-C	8.97%	14.51	3.26E+07	7.51
84	B	5	dry	5D_B	14	ADM-A	8.97%	14.64	1.91E+07	7.28
85	B	5	dry	5D_B	14	ADM-B	8.97%	14.66	1.67E+07	7.22
86	B	5	dry	5D_B	14	ADM-C	8.97%	14.54	1.97E+07	7.29

87	B	5	dry	5D_B	28	ADM-A	8.97%	14.50	2.26E+07	7.35
88	B	5	dry	5D_B	28	ADM-B	8.97%	14.51	2.84E+07	7.45
89	B	5	dry	5D_B	28	ADM-C	8.97%	14.59	2.61E+07	7.42
90	A	5	dry	5D_A	0	ADMA	9.37%	14.53	1.92E+07	7.28
91	A	5	dry	5D_A	0	ADMB	9.37%	14.42	2.03E+07	7.31
92	A	5	dry	5D_A	0	ADMC	9.37%	14.46	1.80E+07	7.26
93	A	5	dry	5D_A	1	ADMA	9.37%	14.47	2.06E+07	7.31
94	A	5	dry	5D_A	1	ADMB	9.37%	14.46	1.51E+07	7.18
95	A	5	dry	5D_A	1	ADMC	9.37%	14.41	2.05E+07	7.31
96	A	5	dry	5D_A	7	ADMA	9.37%	14.44	2.00E+07	7.30
97	A	5	dry	5D_A	7	ADMB	9.37%	14.44	2.09E+07	7.32
98	A	5	dry	5D_A	7	ADMC	9.37%	14.48	1.78E+07	7.25
99	A	5	dry	5D_A	14	ADMA	9.37%	14.46	2.92E+07	7.47
100	A	5	dry	5D_A	14	ADMB	9.37%	14.47	1.05E+07	7.02
101	A	5	dry	5D_A	14	ADMC	9.37%	14.48	9.57E+06	6.98
102	A	5	dry	5D_A	28	ADMA	9.37%	14.41	9.91E+06	7.00
103	A	5	dry	5D_A	28	ADMB	9.37%	14.41	1.04E+07	7.02
104	A	5	dry	5D_A	28	ADMC	9.37%	14.42	1.09E+07	7.04

105	A	30	wet	30W_A	0	AWM-A	37.40%	10.05	4.39E+07	7.64
106	A	30	wet	30W_A	0	AWM-B	37.40%	10.03	4.84E+07	7.68
107	A	30	wet	30W_A	0	AWM-C	37.40%	9.95	3.60E+07	7.56
108	A	30	wet	30W_A	1	AWM-A	37.40%	10.05	1.23E+07	7.09
109	A	30	wet	30W_A	1	AWM-B	37.40%	9.98	1.97E+07	7.29
110	A	30	wet	30W_A	1	AWM-C	37.40%	9.99	1.62E+07	7.21
111	A	30	wet	30W_A	7	AWM-A	37.40%	9.98	6.26E+08	8.80
112	A	30	wet	30W_A	14	AWM-B	37.40%	10.03	7.91E+08	8.90
113	A	30	wet	30W_A	14	AWM-C	37.40%	10.00	6.67E+08	8.82
114	A	30	wet	30W_A	28	AWM-B	37.40%	10.00	8.89E+07	7.95
115	A	30	Wet	30W_A	28	AWM-C	37.40%	10.06	3.47E+07	7.54