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Article

Preharvest Environmental and Management Drivers of Multidrug Resistance in Major Bacterial Zoonotic Pathogens in Pastured Poultry Flocks

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Abstract: Due to nutritional benefits and perceived humane ways of treating the animals, the demand for antibiotic-free pastured poultry chicken has continued to be on a steady rise. However, despite non-usage of antibiotics in pastured poultry broiler production, antibiotic resistance (AR) is reported in zoonotic poultry pathogens. However, actors that drive multidrug resistance (MDR) in pastured poultry are not known. In this study, we used machine learning and deep learning approaches to predict farm management practices, and physicochemical properties of feces and soil that drive MDR in zoonotic poultry pathogens. Antibiotic use in agroecosystems is known to contribute to resistance. Evaluation of the development of resistance in environments that are free of antibiotics such as the all-natural antibiotic-free, pastured poultry production systems described here is critical to understand the background AR. We analyzed 1,635 preharvest (feces and soil) samples collected from forty-two pastured poultry flocks and eleven farms in the Southeastern United States. CDC National Antimicrobial Resistance Monitoring System guidelines were used to determine antimicrobial/multidrug resistance profiles of *Salmonella*, *Listeria* and *Campylobacter*. A combination of two traditional machine learning (RandomForest and XGBoost) and three deep learning (Multi-layer Perceptron, Generative Adversarial Network, and Auto-Encoder) approaches, identified critical farm/environmental variables that drive multidrug resistance in poultry pathogens, in broiler production systems that represents background resistance. This study enumerates management practices that contribute to AR and recommendations to potentially mitigate multidrug resistance and prevalence of *Salmonella* and *Listeria* in pastured poultry.

Keywords: antimicrobial multidrug resistance; foodborne pathogens; food safety

1. Introduction

Increasing incidence of antibiotic resistance (AR) is a major global threat to public health. Apart from the main usage of antibiotics for treatment of infection, there is also prophylactic use to enhance growth in commercial poultry production (Roth *et al.*, 2019). However, persistent presence of antibiotics in the environment could enhance the expression of antibiotic resistance genes and potentiate antibiotic resistant bacteria. In a worst-case scenario, pathogens become resistant to multiple antibiotics/drugs in a phenomenon known as multidrug resistance (MDR). In addition to overuse and misuse of antibiotics, co-selection of resistance genes due to usage of biocides or presence of heavy metals found naturally in the soil in agricultural environments can contribute to AR. Prevalence of MDR poultry pathogens such as *Salmonella spp.*, *Listeria spp.* and *Campylobacter spp.* is a food biosafety concern. Poultry is a major repository of these pathogens, and millions of people depend on poultry products for their daily protein supply. Together, *Salmonella*

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spp., *Listeria spp.*, and *Campylobacter spp.* are responsible for 90 percent of the 9.4 million foodborne illnesses reported in the US (Scallan *et al.*, 2011).

The AR transmission mode and rates between animal, environment, and human, is not well understood. As the potential risk of exacerbating AR in humans is a major concern, global efforts to mitigate AR are required. *Salmonella* MDR in pastured poultry is reported to be comparable to the MDR observed in conventional poultry production systems (Rothrock *et al.*, 2021). However, specific farm management practices and/or physicochemical properties of the preharvest samples (feces, soil) that could contribute to the development of MDR are not known. Here, we sampled eleven different antibiotic-free pastured poultry farms, representing forty-two flocks in the Southeastern United States. Three bacterial pathogens (*Salmonella*, *Campylobacter*, *Listeria*) were isolated from preharvest samples (feces and soil) and AR profiles were characterized utilizing National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) protocols (www.cdc.gov/narms). To determine the management and environmental drivers of MDR among these pathogens, an ensemble machine learning approach comprising both traditional learning and deep learning was employed.

2. Materials and Methods

Forty-two flocks from eleven pastured poultry farms located in the southeastern U.S. were sampled over a period of four years. All broilers flocks were considered all-natural, pasture-raised and never had any antibiotics administered to them during their grow-out, nor were they used historically on the farms.

Sample Collection: Preharvest samples (feces and soil) were collected from the pasture (i) within a few days of broilers being placed on the pasture, (ii) halfway through their time on pasture, and (iii) on the day the flock was processed. At each sampling time, the pasture area was divided into five separate sections, and five subsamples in each section were pooled into a single sample for each section (five total samples each of feces and soil samples were collected on each sampling day). The total volume of sample collected for each field sample was at least 25 g. All samples were collected in the field, and returned to the lab in a cooler packed in ice. Three g (feces, soil) were combined within filtered stomacher bags (Seward Laboratories Systems, Inc.,West cSussex, UK), and diluted 1:3 using 10 mmol/L phosphate-buffered saline (PBS). All samples were homogenized for 60 s and homogenates were used for all downstream cultural isolations.

2.1. Cultural Isolation Methods

Salmonella spp.: As a pre-enrichment step, the stomached homogenates remained in the filtered stomacher bags and were incubated overnight at 35°C. Two different enrichment broths were used to isolate *Salmonella spp.* from these environmental samples: Tetrathionate broth and Rappaport Vassiliadis (Becton Dickinson) media.After overnight incubation at 42°C in both of these enrichment broths, one loopful from each enrichment broth was spread on two different differential media: Brilliant Green Sulfa with novobiocin (Becton-Dickinson) agar and xylose lysine tergitol-4 (Becton Dickinson) agar. These plates were incubated overnight at 35°C, and on each plate, three *Salmonella*-like colonies per subsample were picked and confirmed using triple sugar iron agar (Becton-Dickinson) and lysine iron agar fermentation (Becton-Dickinson) using an incubation period of 18 to 24 h at 35°C. Final confirmation of suspect triple sugar iron/lysine iron agar isolates was performed using *Salmonella* polyvalent O antiserum agglutination (Becton-Dickinson) using the manufacturer's specifications. Positive *Salmonellae* were serogrouped using individual *Salmonella* poly O antisera for O groups A through I following the Kauffman–White scheme (Popoff, 1997).

Campylobacter spp.: Recovery of *Campylobacter spp.* from homogenized samples was performed as previously described (Stern *et al.*, 1992). Initially, 100 mL of homogenized

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suspension was removed, plated onto Campy-Cefex agar, and incubated at 42 ± 1°C for 36 to 48 h in a microaerobic atmosphere (5N2). Putative *Campylobacter spp.* colonies were enumerated, and up to five colonies per sample were subcultured on Brucella agar supplemented with 10% lysed horse blood (BAB plates) for isolation and incubated as previously described.

Listeria spp.: As a pre-enrichment step, the stomached homogenates remained in the filtered stomacher bags and were incubated overnight at 35°C. This pre-enrichment step was followed by two enrichments in UVM Modified *Listeria* Enrichment Broth (Becton-Dickinson) and Fraser Broth (Becton Dickinson), both requiring overnight incubation at 30°C. One loopful of the Fraser's enrichment was streaked for isolation of *Listeria* selective agar (Becton-Dickinson). These plates were incubated overnight at 30°C, and on each plate, three *Listeria*-like colonies per positive subsample were picked and confirmed as *Listeria* using the appropriate BAX PCR assay (DuPont).

2.2. Antibiotic Sensitivity Testing

For all three target bacteria, the published NARMS protocols and NARMS breakpoints were used for characterization and AR determination for each isolate (www.cdc.gov/narms). Isolates were considered multidrug resistant if they were resistant to three or more tested antibiotics.

Salmonella spp.: Recovered isolates were subcultured on blood agar plates (BAPs) overnight at 36 ± 1°C, twice sequentially. One or two colonies were used to inoculate 5 mL of demineralized water to achieve a 0.5 McFarland equivalent using the Sensititre nephelometer (ThermoScientific, TREK Diagnostics, Inc.). After vortexing, 10 mL of the cell suspension was transferred to 11 mL of Sensititre Cation adjusted Mueller-Hinton Broth with TES, followed by thorough vortexing. Fifty microliters of the inoculum was transferred to each well of the Sensititre NARMS Gram-Negative Format CMV3AGNF plate (Trek Diagnostic Systems). These antibiotic sensitivity testing plates contained varying concentrations of the following antimicrobials: cefoxitin, azithromycin, chloramphenicol, tetracycline, ceftriaxone, amoxicillin/clavulanic acid (2:1), ciprofloxacin, gentamicin, nalidixic acid, ceftiofur, sulfisoxazole, trimethoprim/sulfamethoxazole, ampicillin, and streptomycin. Plates were sealed and incubated at 36 ± 1°C for 24 h. Quality control strains *E. coli ATCC25922, Staphylococcus aureus ATCC 29213, Enterococcus fecalis ATCC 29212,* and *Pseudomonas aeruginosa ATCC 27853* were included in susceptibility tests as controls (Clinical and Laboratory Standards Institute, 2010).

Campylobacter spp.: Recovered *Campylobacter spp.* isolates were subcultured on BAPs overnight at 42 ± 1°C. Three or four colonies were used to inoculate 5 mL of Sensititre Cation adjusted Mueller- Hinton Broth with TES (ThermoScientific, Trek Diagnostics, Inc.) to achieve a 0.5 McFarland equivalent using the Sensititre nephelometer. After vortexing, 100 mL of the suspension was transferred to 11 mL of Sensititre Cation adjusted AutoRead Mueller-Hinton Broth with TES and 5% lysed horse blood. One hundred microliters of the inoculum was transferred to each well of the Sensititre CAMPY custom-made microtiter panel as previously described (Chapin and Musgnug 2004, Gupta *et al.* 2004). The panel contains nine antimicrobials: azithromycin, ciprofloxacin, clindamycin, erythromycin, florfenicol, gentamicin, nalidixic acid, telithromycin, and tetracycline. Panels were incubated under microaerobic conditions at 42 ± 1°C for 24 h, and *Campylobacter jejuni isolate 33560* (Konkel and Joens, 1990) was used as a quality control organism.

Listeria spp.: Recovered isolates were subcultured on BAPs overnight at 36 ± 1°C, twice sequentially. Colonies were used to inoculate 5 mL of Sensititre Cation adjusted Mueller–Hinton Broth with TES to achieve a 0.5 McFarland equivalent using the Sensititre nephelometer (ThermoScientific, TREK Diagnostics, Inc.). After vortexing, 50 mL of the cell suspension

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was transferred to 11 mL of Sensititre Cation Adjusted Mueller-Hinton Broth with TES with lysed horse blood, followed by thorough mixing by inversion. One hundred microliters of the inoculum was transferred to each well of the Sensititre NARMS Gram-Positive Format CMV3AGPF plate (Trek Diagnostic Systems). These antibiotic sensitivity testing plates contained varying concentrations of the following antimicrobials: tigecycline, tetracycline, chloramphenicol, daptomycin, streptomycin, tylosin tartrate, quinupristin/dalfopristin, linezolid, streptogramins, nitrofurantoin, penicillin, kanamycin, erythromycin, ciprofloxacin, vancomycin, lincomycin, and gentamicin. Plates were sealed and incubated at 36 ± 1°C for 24 h. A quality control strain (*Streptococcus pneumoniae* ATCC 49619) was included in susceptibility tests as a positive control (Clinical and Laboratory Standards Institute, 2010). Reported minimum inhibitory concentration values were divided by two to compensate for the double volume of inoculum transferred to plate wells, and the NARMS *Enterococcus* breakpoints were used for all drugs except penicillin, which has a *Listeria*-specific breakpoint listed.

2.3. Physicochemical Analysis

Feces and soil samples were analyzed as previously described (Rothrock Jr *et al.*, 2019). The moisture content of the fecal and soil samples was determined by drying overnight at 65°C and calculating the difference between the wet and dried weights of the soil/feces. Fecal and soil pH and electrical conductivity (EC) were determined using an Orion Versa Star Advanced Electrochemistry Meter (ThermoScientific) using a 1:5 dilution in distilled water. Fecal and soil samples were submitted to the University of Georgia Soils Testing Laboratory for determining the elemental composition.

Thirty-one distinct farm variables and management practice variables associated with the feces and soil that were inputs for RandomForest algorithm (Hwang *et al.*, 2020) were used with an ensemble of five different machine learning approaches. Furthermore, the following twenty-four constituents/properties of poultry feces and pastured soil were included as additional input variables for models in this study: acidity/alkalinity (pH), electrical conductivity (EC), moisture, total carbon (TotalC), total nitrogen (TotalN), carbon-to-nitrogen ratio (C:N), aluminum (A), boron (B), calcium (Ca), cadmium (Cd), chromium (Cr), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), molybdenum (Mo), sodium (Na), nickel (Ni), phosphorous (P), lead (Pb), sulfur (S), silicon (Si), and zinc (Zn).

2.4. Pathogen Prediction: Approach and Experiments

An imbalanced, noisy, complex data set presents a major challenge in predicting MDR. This research uses predictive analytics to estimate the likelihood of MDR in *Salmonella*, *Campylobacter* and *Listeria* by using historical poultry farm management variables[1]. We used four-fold cross-validation for all the models to train, evaluate, and predict the data sampled from the dataset. Our analyses are performed by using Python (v3.7.11), Tensorflow (v1.15) (Abadi *et al.*, 2016), Keras (v2.3.1) (Chollet *et al.*, 2015), scikit-learn (v1.0.2) (Pedregosa *et al.*, 2011), pandas (v1.3.4) (Wes McKinney 2010, pandas development team 2020), imblearn (v0.9.0) (Lemaître *et al.*, 2017), and SHAP (SHapley Additive exPlanations) (v0.39.0) (Lundberg and Lee, 2017) libraries. In our predictive modeling, we rely primarily on three processes: 1) standardization or normalization to reduce redundancy. 2) oversampling to balance skewed distribution. 3) deep learning to enhance confidence in MDR prediction.

2.4.1. Standardization

The goal of normalization is to convert numeric values in a dataset to a standardized scale while maintaining the differences in range. We compared the classification performance of four different normalization methods (unit normalization, robust scale

[1] Please visit https://github.com/nispillai/EnsembleModellingForFoodSafety for our code.

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standardization, quantile transformation, and standard scale normalization) with our data. Our results showed that quantile transformations, unit normalization, and robust scale normalization were effective in classification of MDR in *Salmonella*, *Campylobacter* and *Listeria*, respectively. To perform the normalization, we used scikit-learn libraries with default parameters.

a) Unit Normalization: This method normalizes every sample, shrinking/stretching the input feature vector (x) to a unit sphere. This ensures that the vector scales to the unit norm without regard to the distribution of the samples (Eq. 1).

$$X = \frac{x}{||x||} \tag{1}$$

b) Robust-Scale Standardization: This scaler centers and scale each feature independently using the quantile range (IQR: Interquartile Range) to reduce the influence of outliers in the feature set. Instead of considering the mean to standardize the feature, the method uses the median that is less significant to the outliers in scaling.

c) Quantile Transformation: A quantile function provides an approximation of the quantile positions of actual values by inversely calculating the cumulative distribution function. In quantile transformations (Krzysztofowicz, 1997), imbalanced distributions of data with outliers are converted to uniform distributions. This non-linear transformation smoothes out the relation between observations by removing the linear correlation between the input variables. It is a popular and effective way to improve prediction with complex and noisy inputs.

2.4.2. Oversampling

The percentage of negative samples outweighs the percentage of positive samples in our data. Oversampling is a technique often used to balance out such skewed distributions. It maintains class balance by adding new points to the minority class rather than removing them from the majority class. We tested two popular oversampling strategies to balance the distribution during data processing. In our empirical evaluations, random sampling proved effective for *Salmonella* MDR classification, while SMOTE sampling was effective for prediction of MDR in *Campylobacter* and *Listeria*. To perform these oversampling approaches, we used the imblearn library with default parameters.

a) Random Oversampling: In random oversampling (Lemaître *et al.*, 2017), an increase in sample size is achieved by selecting minority class examples in random order and including them in the training set. This sampling technique iterates until a majority sample equals a minority sample.

b) Synthetic Minority Oversampling Technique: SMOTE (Chawla *et al.*, 2002) is based on the selection of a random sample from the minority class and one of its nearest neighbors, followed by the generation of a new synthetic sample within that range. Oversampling uses the nearest neighbor method in place of adding random duplicate samples to the minority class.

2.4.3. Deep Neural Network Learning

We compared the performance of three different deep learning architectures to find an efficient method of detecting MDR for *Salmonella*, *Campylobacter*, and *Listeria*. In the first step, we handle outliers with standardization techniques, then oversample to equalize the distribution, and use deep learning to identify the best prediction parameters. For *Listeria* MDR, a generative adversarial network (GAN) proved more effective than autoencoding, while a multi-layer perceptron (MLP) provided the best results for *Campylobacter* MDR.

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Additionally, *Salmonella* MDR classification was best performed with the auto-encoder design. In order to implement the following neural network models, we used Python, TensorFlow, and Keras libraries.

a) Multi-Layer Perceptron: An MLP (Haykin, 1994) is a type of feed forward artificial neural network that can distinguish data that cannot be linearly separated. These multi-layered networks consist of hidden nodes with a non-linear activation function that are connected with specific weights to the next layer of nodes. At the learning stage, connection weights are adjusted based on the amount of error in the output using a backpropagation function. We used 30 neural network units in the hidden layer, along with a binary classification output layer. The weights of our kernels are initialized with he uniform (He *et al.*, 2015) and Adam (Kingma and Ba, 2014) algorithms for stochastic gradient descent.

b) Generative Adversarial Network: A generative adversarial network (Goodfellow *et al.*, 2014), or GAN, is a deep learning method for generating models from data using supervised learning techniques. Generative modeling involves discovering and understanding regularities and patterns in data. Rather than treating the problem as an unsupervised problem, GAN treats it as a supervised problem with two submodels: a generator model and a discriminator model. The generator model attempts to generate new samples from the negatives, while the discriminator model tries to determine what is positive and what is negative. Using backpropagation, we train the generator and discriminator models together. The discriminator model is composed of a hidden layer with 50 units and an output node. In the generator model, there are three layers: a hidden layer of 70 units, a latent layer of 50 units, and a generator layer of 70 units. We compute the results using the ReLU activation function and Adam optimizer, as well as he uniform kernel initialization.

c) Auto-Encoder: Autoencoder (Liou *et al.*, 2014) is a stacked neural network layer system comprising an encoder layer, a latent or representative layer, a decoder layer, and an output layer. The latent layer will embed data without labels in a meaningful manner, and the output layer will attempt to recreate the original input. With the backpropagation algorithm, the networks are learned by minimizing the reconstruction error, which is the difference between the original and the reconstructed inputs. Our model consists of an encoder layer and a decoder layer with 70 neural network units each and a latent layer with 30 neural network units. Additionally, we have a prediction model using 70 neural network units and a binary output node that can predict positive and negative samples from the latent representation. We use he uniform for the initialization of our kernel weights, Adam for stochastic gradient descent, and ReLU for activation.

2.5. Critical Farm Feature Selection

In order to determine the critical and influential features in farming practices associated with MDR, we use a multi algorithm ensemble approach. Besides the three deep learning algorithms (MLP, GAN, and Encoder), we also use popular machine learning algorithms like Random Forest and XGBoost in this ensemble model. Following the training of five learning models with the same data, we use the SHAP library (Lundberg and Lee, 2017) to select the top 15 important features from all models. We use majority voting i,e, features selected by at least three models to determine the most influential features.

a) Random Forest: In RandomForest (Ho, 1995), individual decision trees that work together make up an ensemble of powerful algorithms. Using random samples from the dataset with replacement, random forest constructs several decision trees and predicts the outcome based on the majority vote.

b) eXtreme Gradient Boosting: XGBoost (Chen and Guestrin, 2016) is another powerful decision-tree-based ensemble technique that incrementally improves the performance by

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adding new models in sequence to fix previous models' errors. The gradient descent optimization algorithm minimizes the weak prediction loss of current models when adding new models.

SHapley Additive exPlanations: SHAP (Lundberg and Lee, 2017) allows us to compute the effect of each feature on the model output. A model's output with and without a specific feature is compared to determine its relative importance. When SHAP values are positive, they indicate greater importance than when SHAP values are negative. Feature values with negative SHAP values are recommended to lower the presence of multidrug resistance.

3. Results and Discussion

There is growing interest in antibiotic resistance (AR) and multidrug resistance (MDR) in the agricultural sector as they are public health concerns especially for both clinicians and veterinarians. Use of antibiotics for treatment of animal infections and prophylactic use at sub-therapeutic dose to enhance animal growth could lead to MDR and transmission to humans. About 148 mg of antibiotics per kilogram of chicken is estimated to be consumed by humans and the quantity is projected to continue in upward direction (Van Boeckel *et al.*, 2015). Application of animal waste as organic manure is an indirect environmental mechanism of AR transmission to humans as these anthropogenic contaminants could potentially enhance antibiotic resistance genes of the microorganisms in the ecosystem (Manyi-Loh *et al.*, 2018). Nevertheless, microorganisms such as *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, and *Enterococci* possess intrinsic capability to develop antibiotic resistance without encountering any antimicrobial contaminant (Reygaert 2018, Pang *et al.* 2019). Recent studies report the prevalence of MDR (resistance to three or more antibiotics) isolates of *Salmonella*, *Listeria* and *Campylobacter*, important zoonotic pathogens on poultry farms without historical or exogenous sources of antibiotics (Rothrock Jr *et al.*, 2016). However, the drivers of MDR from the environment (soil) or animals themselves (feces) are yet to be identified. We utilized an ensemble approach combining machine learning and deep learning to identify preharvest management practices that are predictive of MDR in poultry pathogens.

Table 1 is an overview of the number of samples in our dataset that exhibit varying degrees of antibiotic resistance.

Table 1. Sample type and the number of samples identified as multidrug resistant (MDR, resistance to $\geq$ 3 antibiotics tested) of *Salmonella* (S_MDR), *Campylobacter* (C_MDR), and *Listeria* (L_MDR)

	Sample Type	Resistance to 3 or more antibiotics	Resistance to 1 or 2 antibiotics	Resistance to 0 antibiotics
Salmonella_MDR	Feces	31	52	31
	Soil	31	29	23
	Overall Preharvest	62	81	54
Campylobacter_MDR	Feces	16	72	255
	Soil	8	10	132
	Overall Preharvest	24	82	387
Listeria_MDR	Feces	73	28	0
	Soil	97	17	0
	Overall Preharvest	170	45	0

Only 27% of fecal samples had *Salmonella* resistant to three or more antibiotics (MDR), while 37% *Salmonella* isolates were MDR in soil samples. *Salmonella* MDR included antibiotics that inhibit protein synthesis (tetracycline, streptomycin) and antibiotics that target cell wall synthesis (ampicillin, augmentin, cefoxitin, ceftriaxone, ceftiofur) among the antibiotics tested in this study. Our results also show that the majority of the *Salmonella* isolates with MDR do not cause human infections (data now shown) consistent with earlier observation (Rothrock *et al.*, 2021).

Campylobacter MDR (C_MDR) samples are highly imbalanced i.e., the percentage of negative samples outweighs the percentage of positive samples with only 4% posi-

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tives in feces and 5% in soil. C_MDR included antibiotics that inhibit protein synthesis (Azithromycin, gentamicin, clindamycin, erythromycin, florfenicol, telithromycin, tetracycline) and antibiotics that target DNA replication (Ciprofloxacin and nalidixic acid).

The MDR observed in this study for *Listeria* in feces and soil samples is 72% and 85% respectively. *Listeria* MDR predominantly involves antimicrobials that inhibit protein synthesis such as tetracycline, streptomycin, daptomycin, lincomycin, streptogramins and tigecycline. Resistance to ciprofloxacin that inhibits DNA replication was also observed. MDR rates reported here corroborate the reported rates of 36.0%, 1.4%, and 63.9% in *Salmonella*, *Campylobacter*, and *Listeria*, respectively in a survey of six pastured poultry farms (Rothrock Jr *et al.*, 2016). Our study examined preharvest feces, soil, as separate models and also a pre-harvest common model (combined soil and feces) for MDR classification with *Salmonella*, *Campylobacter*, and *Listeria*. Our ensemble approach utilized two traditional machine learning (RandomForest (RT) and eXtreme Gradient Boosting (XG)) and three deep learning (Multi-layer Perceptron (MLP), Generative Adversarial Network (GAN), and Auto-Encoder (ENC)) methods.

For each of these classifications, the prediction confusion matrix which is used to compare the model performance is shown in Table 2.

Table 2. The classification results of MDR predictive preharvest (feces and soil) models. Models are trained with farm practice and environmental physicochemical variables

	Sample Type	Actual	Predicted +	Predicted -	Precision	Recall	Specificity	F1-Score
Salmonella_MDR	Feces	+	16	1	0.88	0.94	0.83	0.9
		-	3	14				
	Soil	+	9	2	0.98	0.83	0.98	0.89
		-	0	10				
	Overall Preharvest	+	27	0	0.93	0.98	0.92	0.95
		-	2	25				
Campylobacter_MDR	Feces	+	65	0	0.94	1	0.94	0.97
		-	4	62				
	Soil	+	28	1	0.89	0.97	0.87	0.93
		-	4	25				
	Overall Preharvest	+	93	1	0.92	0.98	0.92	0.95
		-	8	86				
Listeria_MDR	Feces	+	12	3	0.94	0.80	0.95	0.86
		-	1	14				
	Soil	+	16	3	0.96	0.82	0.96	0.88
		-	1	19				
	Overall Preharvest	+	28	6	0.92	0.83	0.92	0.87
		-	3	31				

In order to avoid overfitting in our models, the scores are averaged from four-fold stratified cross-validation. Results show that the models had a large proportion of false positives and false negatives due to the imbalanced input dataset. In summary, our learning models are capable of predicting MDR with greater than 86% confidence. Additionally, we generated receiver operating characteristic curves (ROC curves), to show the performance of our binary classification models at different thresholds. Figure 1 shows representative ROC curves in *Listeria* models.

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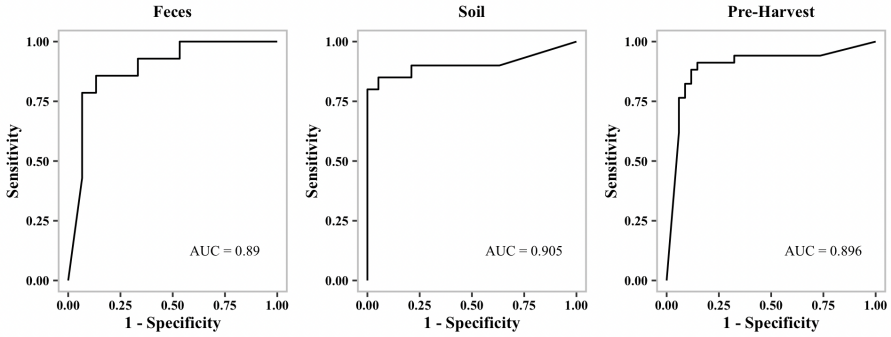

Figure 1. Receiver operating characteristic curve (ROC curve) of *Listeria MDR* predictive models.

The influential variables for the pathogens MDR are enumerated in the following Tables 3, 4, and 5 as determined by our ensemble approach.

Table 3. The influential variables and their rank in respective algorithm for the prediction of *Salmonella* multidrug resistance (S_MDR). The ***top five*** ranks are shown in ***blue*** and the ***top ten*** in ***brown***. Overall ***top five*** most influential variables for *S_MDR* in feces are Mg, Water_Source_Well, Pasture_Housing_CTF, pH and EC while the ***top five*** for soil are Mg, P, Na, EC and Ca.

SampleType	Model	Mg	P	K	Fe	EC	pH	Mn	Na	WaterSource Well	Pasture Housing_CT	Flock Size	Ca	Flock AgeDays	PastureHousing_CTF	BrSoy Free
Feces	RT	1				5				8	6			15		
	XG					14	3		2	4		8		1		
	MLP	5	1	3		4		6		10	9		2			
	ENC		4		1		10	5	3			2		7	11	
	GAN	4					8			6	5					
Soil	RT						7	10				9			6	5
	XG	2				10							7	1		
	MLP		3	5	6		10	8	2			4	1		12	
	ENC	2	3	5		1		8	6			4		7	10	
	GAN	4	2		9	3			5	10	12		6	7		

Table 4. The influential variables and their rank in respective algorithm for the prediction of *Campylobacter* multidrug resistance (C_MDR). The ***top five*** ranks are shown in ***blue*** and the ***top ten*** in ***brown***. Overall ***top five*** most influential variables for *C_MDR* in feces are P, EC, Zn, Cu and Mn while the ***top five*** for soil are EC, K, FlockSize, Mg and P

SampleType	Model	P	EC	Mg	K	FlockSize	Mn	C:N	FlockAgeDays	Zn	Cu	Fe	Pb	EggSource	YearsFarming	BroodFeed
Feces	RT		1		13	9	6	8	7					10		
	XG									3	1			15		7
	MLP	1	4	3	6	8		7	5		9	2				
	ENC	2	3		5		4	8	10	6	7				9	14
	GAN	4	6	3	5		10		7	8		2			14	15
Soil	RT		1	4				2						13		
	XG		3	4	5							2		6		7
	MLP	3	1	4	2	5			9	8				11	14	
	ENC	4			3	1		6	2							
	GAN	5		1	2	3				4	6				8	

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Table 5. The influential variables and their rank in respective algorithm for the prediction of *Listeria* multidrug resistance (L_MDR). The ***top five*** ranks are shown in ***blue*** and the ***top ten*** in ***brown***. Overall ***top five*** most influential variables for *L_MDR* in feces are K, P, FlockSize, Cu and Cr while the ***top five*** for soil are K, FlockSize, Mg, C:N and YearsFarming

SampleType	Model	K	FlockSize	P	Mg	EC	AvgNumBirds	Cr	Cu	C:N	Na	Cd	YearsFarming
Feces	RT	1		3	7	6		8	4	10	5		2
	XG		5									15	
	MLP	1					2	10	6		5		
	ENC	3	5	2			4			8		15	
	GAN	3	2	1		6		9	5				8
Soil	RT			5				7	2	8	10	11	15
	XG												
	MLP	3	1		4					5			
	ENC	2	5		4		3						12
	GAN	2	1		5				8	7	4		10

Tables 3 (*Salmonella* MDR), 4 (*Campylobacter* MDR), and 5 (*Listeria* MDR) show the most important feature variables and their rankings in individual algorithm predictions. These results indicate that magnesium (Mg), phosphorus (P), electrical conductivity (EC), flock size, and potassium (K) are important factors for MDR prediction in all three pathogens.

While Mg, P, and flock size appear to affect MDR in all pathogens, both *Salmonella* and *Campylobacter* MDR are further affected by EC, Mn, flock age and both Listeria and *Campylobacter* MDR appears to be affected K, C:N, and Cu. *Salmonella* MDR alone is affected by pH, Na, Ca, water source, housing type. *Campylobacter* MDR alone is affected by Zn, Egg source, brood feed and *Listeria* MDR alone by Cr and years of farming (Tables 3, 4, and 5). However, as shown in Table 1 and reported earlier, prevalence of *Campylobacter* MDR is negligible in the pastured poultry farms included in this study. Therefore, the focus of this study will be on *Salmonella* and *Listeria* MDR in this manuscript.

SHAP dependency plots show the potential internal factors from feces that drive *Salmonella* MDR, such as EC and pH, while the external factors determined from the soil include P, Mn, and Na (Table 6). Only Mg appears to be critical for *Salmonella* MDR in both feces and soil. In *Listeria*, P, Cu and Cr are correlated to MDR in feces while Mg, C:N, and years of farming are identified to be important for MDR in soil (Table 7). Flock size and K appear to be critical for *Listeria* MDR in both soil and feces models. Although the exact mechanisms by which these farm management practices and physicochemical properties impact MDR are not fully understood, the following section provides possible insights into the impact of variables on *Listeria* and *Salmonella* MDR.

Table 6. Pre-harvest model variable recommendations for *Salmonella* multidrug resistance.

Farm Variable	Salmonella MDR	
	Feces	Soil
Mg	≥ 2000	≤ 300
P		≤ 150
EC	≤ 2000	
pH	≤ 6.5	
Mn		≥ 70
Na		< 50

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Table 7. Pre-harvest model variable recommendations for *Listeria* multidrug resistance.

Farm Variable	Listeria MDR	
	Feces	Soil
Mg		≤ 300
P	≤ 5000	
FlockSize	$\not< 200$	$\not> 700$
K	7000 - 12000	> 200
C:N		> 15
Cu	> 18	
Cr	> 3	
YearsFarming		6 - 15

3.1. Drivers Of Listeria MDR In Pastured Poultry

Possible mechanisms by which preharvest variables, specifically physicochemical properties of soil contribute to the development of MDR based on current literature is discussed below.

Magnesium (Mg): The Mg ion provides a strong cohesive force that strengthens bacterial ribosomes, the protein synthesis machinery. Its presence provides stability for the cell and counteracts the action of ribosome targeting antibiotics. Diminished levels of Mg have been shown to promote the activities of antibiotics and hamper the protein synthesis in bacteria (Dong-yeon *et al.*, 2019). Presence of Mg has also been reported to inhibit the transcription of genes involved in biofilm formation and promote the penetration of antibiotics (Ben-Ishay *et al.*, 2017). In addition, Mg itself has been shown to have antimicrobial properties (Demishtein *et al.*, 2019). While Crippen *et al.* (2016) reported the Mg level of conventional poultry soil to be between 285 and 463 ppm, our analysis suggests optimum level of Mg to prevent MDR as less than 300 ppm. Pastured poultry farmers could utilize gypsum, a calcium sulphate salt that could reduce soil Mg by displacing the Mg within the soil with calcium.

Phosphorus (P): The buffering effect of phosphate formed from phosphorus in the bacterial culture medium has been reported to both enhance and diminish antimicrobial activities depending on the antimicrobial agents. Phosphate promotes resistance of *S. lactis* to streptomycin but increases its sensitivity to tetracycline (Sinha, 1985). Also, addition of 30 to 300ug/L phosphorus has been shown to significantly increase biofilm formation in drinking water supply (Fang *et al.*, 2009) which could potentially increase antibiotic resistance. Reducing the phosphorus level to below 5000 ppm as suggested by this study could potentially reduce MDR.

Potassium (K): Acesulfame potassium is an artificial sweetener found in various consumables and products such as soft drinks, jellies, beverages and also poultry feed. Recent work of Li *et al.* (2022) shows that with increasing concentration of acesulfame potassium, and invariably potassium, the growth of bacteria with antibiotics resistance genes is inhibited. Contrarily, Gries *et al.* (2013) show that uptake of K is essential for growth and antibiotic resistance in *Staphylococcus aureus*. While potassium levels in the conventional poultry litter and soil are reported to be between 3000 and 13000 ppm, our models indicate that potassium levels between 7000 and 12000 ppm is optimal to reduce the incidence of *Listeria* MDR. This supports the observation of Li *et al.* on the detrimental effects of potassium on antibiotic resistance bacteria.

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Carbon Nitrogen Ratio (C:N): Composting is one important way to remove undesirable antibiotics that are not metabolized by animals and get into the environment through excretion. The C:N levels positively correlate and are indicative of the presence of antibiotic resistance genes (ARGs) in the environment (Wei *et al.*, 2020). Our predictive models recommend a C:N higher than 15 to mitigate MDR in *Listeria*. However, recent work of Zhu *et al.* (2021) shows that C:N of 26 is sufficient to remove ARGs from compost, and values higher than this may not be effective. Therefore, C:N values between 15 and 26 ppm are recommended to reduce MDR.

Copper (Cu): Based on Cu exposure studies with 96 microorganism isolates, Cu lead to increased resistance to different clinically important antibiotics (Glibota *et al.*, 2019). However, comprehensive review on the effect of antibiotic-binding capability of Cu indicates that it could both enhance or diminish antibiotic resistance (Poole, 2017). This is not surprising as Cu is an important cofactor for many enzymes required by bacteria. However, at elevated levels it becomes toxic and acts as an antimicrobial and invokes adaptive response in the form of resistance from the organism (Lemire *et al.*, 2013). Our prediction algorithms recommend Cu level greater than 18 ppm to combat MDR.

Chromium (Cr): Investigation into the role of Cr in *Staphylococcus aureus* and *Escherichia coli* establish that it acts as an antibiotic and works synergistically with conventional antimicrobial agents to induce oxidative stress in the organisms (Páez *et al.*, 2013). Nevertheless, bacteria such as *Pseudomonas aeruginosa* have developed effective ways of reducing and ejecting chromium from the cell thereby making it less potent. Increasing the level of chromium to > 3 ppm as predicted by our models could help prevent MDR in *Listeria*.

3.2. Drivers Of Salmonella MDR In Pastured Poultry

Magnesium (Mg): Similar to the observation with *Listeria*, Mg appears to be an important driver of MDR in *Salmonella*. Mg is important for stabilizing protein synthesis machinery. It is interesting to note that both *Salmonella* and *Listeria* exhibit MDR to classes of antimicrobials that target protein synthesis such as aminoglycosides, macrolides, glycylcycline, and ketolides. While the median range in conventional poultry for Mg is around 374 ppm, our prediction models recommend Mg levels lower than 300 ppm to destabilize bacterial ribosomes and increase the pathogen sensitivity to the antibiotics. Furthermore, the role of Mg in modulating nitrosative stress has been established (Bourret *et al.*, 2017). Reduction in the Mg levels proposed in this study may lower the *Salmonella* viability and increase its susceptibility to antimicrobial activity.

Phosphorus (P): P is capable of modulating buffering capacity of the pathogen environment as well as capability to form biofilms thereby potentially altering the sensitivity to antibiotics. Therefore, reducing access of *Salmonella* to phosphorus could reduce the MDR. Our model suggests P less or equal to 150 ppm in the soil could help reduce *Salmonella* MDR. In an in vivo study, dietary and systemic increase in the level of phosphorus has been reported to lower pig mortality rate during *Salmonella* infection (Doak *et al.*, 1972), possibly due to phosphorus mediated stimulation of leukocyte production and defense mechanisms against the pathogen. Therefore, while P restriction in the poultry soil where *Salmonella* is found may be a good practice, supplementation of the poultry brood feed with P may enhance immune response and have synergistic effect in further lowering the *Salmonella* presence and MDR.

Electrical Conductivity (EC): Treatment of MDR methicillin-resistant *Staphylococcus aureus* (MDR-MRSA) with chlorhexidine acetate nanoemulsion has been shown to have both in vivo and in vitro efficacy against the pathogen that correlated with an increase in the electrical conductivity (Song *et al.*, 2016). ECs that range from 0.97 to 10.07 S/m, i.e 970 mS/m to 10,070 mS/m, in graphene oxide have been reported to promote wound healing against MDR-MRSA. While EC levels detected in the samples in this study are below 1,000 μS/cm, our models suggest that soil EC of 2,000 μS/cm (200 mS/m) would be sufficient to reduce MDR incidence in *Salmonella*.

pH: *Salmonella* is equipped with mechanisms to survive a wide range of pH between 4.4 and 9.0 with an optimum pH of around 7.0 (Suehr *et al.*, 2020). Food safety regulation indicates acidic pH of 4.2 or lower is effective against *Salmonella* (Keerthirathne *et al.*, 2016). Recent work of Roy *et al.* (2021) also indicate that pH between 4.0 and 6.0 is sufficient to inhibit biofilm formation in *Salmonella*. Formation of biofilm is known to protect *Salmonella* from both in vitro and in vivo action of antibiotics (González *et al.*, 2018). Our predictive model recommendation for soil pH suggests that a pH 6.5 could reduce MDR possibly by inhibiting biofilm formation.

Manganese (Mn): Mn complex with antibiotic colistin has been reported to be effective against poultry avian pathogenic *Escherichia coli* known for being highly antibiotic resistant. Mn in the metal complex form alone ([Mn(CO)3(tpa-k3N)]Br) has antimicrobial activity and synergistic combination with colistin further increases the killing efficiency of Mn towards the pathogen (Betts *et al.*, 2017). The recommendation here to increase the Mn levels up to 70 ppm or above in the soil compared to 5 to 7 ppm found in the conventional poultry soil (Crippen *et al.*, 2016) could increase its availability and ultimately its antibacterial activity.

Sodium (Na): Presence of Na in the form of sodium chloride salt has been established to both increase the thermal and antibiotic resistance in multiple strains of *Salmonella* (Yoon *et al.*, 2013). This was speculated to be a result of an increase in osmotic stress. Our predictive models suggest that decreasing the Na content of the soil to below 50 ppm could mitigate *Salmonella* MDR

Others: Factors such as water source, housing type, Flock size and age are identified as variables that could contribute to *Salmonella* MDR. However, the observational study described here does not account for factors that may influence such dynamic management practices. Future experiments that control for these changing variables are warranted.

4. Conclusion

In conclusion, while previous work established the presence of AR in pastured poultry with no historical use of antibiotics, in this study, we used machine learning and deep learning approaches to predict farm management practices, and physicochemical properties of feces and soil that drive MDR in zoonotic poultry pathogens *Salmonella*, *Campylobacter*, and *Listeria*. Antibiotic use in agroecosystems is known to contribute to resistance. Evaluation of the development of resistance in environments that are free of antibiotics such as the all-natural antibiotic-free, pastured poultry production systems described here is critical to understand the background AR and its contribution in the animal-environment-human triad. Understanding the drivers of background AR will aid future agroecosystem studies to determine the impact of antibiotic use in animal, environment and public health domains for designing optimal animal production systems to reduce AR in zoonotic pathogens, ensuring safety of animal-based food products.

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Abbreviations

The following abbreviations are used in this manuscript:

AR	Antibiotic resistance
MDR	Multidrug resistance
CDC	Centers for Disease Control and Prevention
RT	Random Forest
XG	eXtreme Gradient Boosting (XGBoost)
MLP	Multi-layer Perceptron
GAN	Generative Adversarial Network
ENC	Auto-Encoder
SHAP	SHapley Additive exPlanations
SMOTE	Synthetic Minority Oversampling Technique
S_MDR	*Salmonella* multidrug resistance
C_MDR	*Campylobacter* multidrug resistance
L_MDR	*Listeria* multidrug resistance
MDPI	Multidisciplinary Digital Publishing Institute
Mg	Magnesium
P	Phosphorus
K	Potassium
Fe	Iron
EC	Electrical Conductivity
pH	Potential of hydrogen
Mn	Manganese
Na	Sodium
Ca	Calcium
C:N	Carbon Nitrogen Ratio
Zn	Zinc
Cu	Copper
Pb	Lead
Cr	Chromium
Cd	Cadmium

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