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Reservoirs and Within-Host Dynamics of Antibiotic Resistance Associated with Small-Scale Poultry Production in Northwest Ecuador

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Reservoirs and Within-Host Dynamics of Antibiotic Resistance Associated with Small-Scale Poultry Production in Northwest Ecuador

By

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An abstract of
A thesis submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Master of Science in Population Biology, Ecology and Evolution
2015

Abstract

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Small-scale poultry production that includes non-therapeutic use of antibiotics is becoming increasingly common in developing countries, contributing to an environmental reservoir of antibiotic-resistant (AR) bacteria and posing a threat to human health. We conducted a multifactorial experiment in a poultry farming facility to assess the effect of non-therapeutic antimicrobial use and high stocking density on the within-host dynamics of resistant and susceptible *Escherichia coli* in broiler chickens. To validate conclusions from the experiment, we used data from an observational study in 17 remote communities in Northwest Ecuador, which allowed to assess the potential for transmission of AR between different ecological reservoirs, including 'production birds' (broiler chickens and laying hens raised for sale), coop surfaces and soil, household soil and drinking water. In both studies we found high levels of resistance associated with production birds, and evidence suggesting that AR strains associated with poultry production have a source external to our study system. The effect of management practices on the ecology of AR in *E. coli* was limited.

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ACKNOWLEDGEMENTS

This work was supported by National Institute of Allergy and Infectious Diseases Grants # R01AI050038 and 1K01AI103544, and the National Science Foundation Ecology and Evolution of Infectious Diseases program Grant #08119234.

I would like to thank the Ecología, Desarrollo, Salud y Sociedad (EcoDESS) project research team for their invaluable contributions to data collection and lab personal at Universidad San Francisco de Quito and University of Michigan for their efforts in sample processing. I am also grateful to my advising committee and my mentors and peers at the Population Biology, Ecology and Evolution Program for their support.

RESERVOIRS AND WITHIN-HOST DYNAMICS OF ANTIBIOTIC RESISTANCE ASSOCIATED WITH SMALL-SCALE POULTRY PRODUCTION IN NORTHWEST ECUADOR

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CHAPTER SUMMARY

Small-scale poultry production that includes non-therapeutic use of antibiotics is becoming increasingly common in developing countries, contributing to an environmental reservoir of antibiotic-resistant (AR) bacteria and posing a threat to human health. We assessed the potential for transmission of AR from 'production birds' (broiler chickens and laying hens raised for sale) to the surrounding environment in Northwestern Ecuador. We sampled 300 production birds, and 455 'household birds' (raised for domestic use) from 291 households in 17 villages between 2010-2013. We also sampled drinking water, household soil and food preparation surfaces, coop surfaces and soil, and surveyed water, sanitation and antibiotic use practices. Up to three *E. coli* isolates per sample were tested against 12 antibiotics. We observed: 1) high levels of AR overall, particularly in production birds, which had over 60% of isolates resistant to tetracycline and sulfonamides, and significantly more AR than household birds ($p<0.01$); 2) a phenotypic resistance pattern to amoxicillin/clavulanate, cephalothin, cefotaxime and gentamicin particular to isolates from production birds and coop surfaces. The prevalence of this signature pattern of AR associated with poultry production declined with bird age and was associated with a particular purchase site. 3) Higher prevalence of resistance for all antibiotics tested in coop versus household samples ($p<0.01$). No difference in AR profiles was observed between

[1] Appears as Braykov NP, Eisenberg J, Grossman M, Zhang L, Cevallos W, Vasquez K, Muños D, Marrs Currently in preparation for submission to Antimicrobial Agents and Chemotherapy

water samples from farming versus non-farming households, or across villages with different farming intensity. These results suggest that AR strains associated with poultry production likely originate from outside sources and are passed to the immediate environment.

INTRODUCTION

Antibiotic resistance (AR) is a growing public health concern in the United States (US Centers for Disease Control and Prevention 2013) and globally(World Health Organization (WHO) 2014).The overuse and misuse of antimicrobials in human medicine (Turnidge and Christiansen 2005; Bergman et al. 2006) and in animal agriculture, where the vast majority of antimicrobials are used in the production of food animals for purposes of growth promotion and disease prevention (Sarmah, Meyer, and Boxall 2006), contributes to the evolution and spread of resistant pathogens (Silbergeld, Graham, and Price 2008). Although the mechanism that links human resistance and agriculture remains to be fully elucidated (Phillips et al. 2004; Marshall and Levy 2011a) it is known that farmed animals and the broader environment, particularly soil, serve as reservoirs of antimicrobial resistance genes that can be exchanged across species (Forsberg et al. 2012; Baquero, Tedim, and Coque 2013; Perry and Wright 2013; Finley et al. 2013). Numerous resistance genes in human pathogens have been found to have environmental origins (Wright 2010), and the environmental resistome is enriched and mobilized when soil and water are contaminated with runoff from farms (Davis et al. 2011), or antibiotic residues (Ding et al. 2014; Sarmah, Meyer, and Boxall 2006). Multiple pathways link resistance in these reservoirs to human health: epidemiological studies going back to the 1970s show an association between antibiotic use on farms and colonization with livestock-associated strains in workers (S. B. Levy, FitzGerald, and Macone 1976) and surrounding communities (Neyra et al. 2014). Wide attention has been paid to direct pathogen transmission through the food chain (Barton 2014).

The non-therapeutic use of antibiotics for growth promotion has been banned in all European Union countries since 2006 (Castanon 2007), and the United States is also working towards phasing out the practice on precautionary grounds (US Food and Drug

Administration 2013). However, in many developing countries, non-therapeutic use of antibiotics in animal husbandry has continued, and has even been promoted as a strategy to address under-nutrition (Angulo and Collignon 2005). Poultry production is rapidly growing worldwide and is a heavy user of antibiotics to enhance growth and prevent disease (Dibner and Richards 2005). Backyard poultry farming that employs growth promotion is advanced as a development strategy in Africa, Asia and Latin America (Agricultural Research Council 2010; Food and Agriculture Organization 2011). Given the sustained global increase in poultry and concurrent use of agricultural antibiotics, there is a need for more ecological studies examining connections between animals in intensive production, the surrounding environment and potential human health impacts, especially where animals are raised in close proximity to human communities.

In this study we examine the link between animal husbandry and environmental reservoirs of AR in a field site in rural Ecuador, where small-scale poultry farming has been promoted by development agencies. We take advantage of an ongoing study in Northwest Ecuador where we have been carrying out research on the transmission of diarrheal diseases since 2003 (Eisenberg et al. 2012; Eisenberg et al. 2006). We compared AR in the poultry production environment, including samples from production birds (broilers and laying hens raised for sale) and coop soil and surfaces, versus the domestic environment, including samples from household birds raised for domestic use, household drinking water, and household soil and surfaces. We isolated *Escherichia coli* as a sentinel organism to examine phenotypic patterns of resistance in the intensive production versus domestic environment. Our study integrates these epidemiological data with additional qualitative (ethnographic) information to characterize poultry production in the region, and set a foundation for further ecological modeling of the contribution of poultry agriculture to the development of AR in humans.

METHODS

Study area

The study was conducted between August 2010-July 2013 in a remote region of Esmeraldas Province, in northern coastal Ecuador populated primarily by Afro-Ecuadorians, as well as the Chachi indigenous group, and people of mixed ethnicity. Community members primarily consume untreated surface source water and sanitation facilities are inadequate (K. Levy et al. 2012). Our research team has been working in a total of 31 communities in this region since 2003; the study sites and region are described in detail elsewhere (Eisenberg et al. 2012; Eisenberg et al. 2006). The present study was carried out in a subset of 17 villages where we were able to collect environmental and animal samples.

Previous research has shown that the observed antibiotic resistant patterns when comparing villages of varying remoteness to a road was driven by transmission rates of resistant bacteria, the introduction of antibiotic resistant bacteria to villages of the region from outside sources, and human antimicrobial use (Eisenberg et al. 2012). The new road has increased access to resources, including antibiotics, different poultry breeds suitable for intensive farming, feed and veterinary drugs, and has led to the rise of household-level commercial poultry farming. Production is also supported by foreign and local development authorities as an inexpensive source of protein and a tool to eradicate poverty.

Ethnography

To understand the context of the sampling and biological analysis, ethnographers conducted structured interviews with local stakeholders, including poultry farmers, veterinary pharmacy owners, hatchery owners, and community leaders. Information was

gathered on the practices, organization, economics and history of poultry farming, including antibiotic use, rearing and consumption of different kinds of birds..

Household surveys

We visited villages monthly to record the total number of production birds in each community. In addition, we conducted a detailed survey within 10 days of sample collection on the type, size, age, origin and intended use of flocks, the brand and types of feed used, and supplementation with antibiotics.

Informed consent was obtained from all participating households. The Institutional Review Boards of the University of Michigan, Universidad San Francisco de Quito, Trinity College, and Emory University approved all interaction with human subjects.

Poultry feed samples

We collected seven samples of the most commonly sold brand of feed (Nutril) from a veterinary store in the central trading city of Borbón in July 2009. Every type of feed sold was sampled, including starter, fattening, and finishing feed for broilers and layer hens. Chemical extractions were carried out at Emory University laboratories in April 2010, and mass spectrometry analysis was carried out in the lab of John Barr at the U.S. Centers for Disease Control and Prevention. Samples were tested for the presence of the following antibiotics: lincomycin, virginiamycin, bacitracin, flavomycin, avilomycin, tylosin, nitrofurantoin, chloramphenicol, tetracycline, sulfamethazine, sulfathiazole. With the exception of nitrofurantoin, the first 8 drugs in the panel belong to antibiotic classes that are exclusively used to treat and prevent infections with Gram-positive bacteria, and were therefore not included in susceptibility testing of *E. coli* isolates.

Sample collection & laboratory analysis

In villages with production poultry farming, teams visited 1) all households with active poultry coops, and 2) an equivalent number of non-farming households that were located as far as possible from coops. If villages had no poultry farming at the time of the visit, a minimum of three, and maximum of ten, non-farming households were chosen at random, depending on the size of the village.

Poultry samples

Two types of birds were sampled in the communities. 'Production birds' included breeds of laying hens raised for egg production or broiler chickens that are raised in coops for 6-7 weeks before slaughter, eating formulated feed containing antibiotics and produced by poultry suppliers. In addition to the antibiotics pre-mixed in the feed, birds are commonly given antibiotics as prophylaxis via water. Capacity of coops ranged from ~50-100 birds of a single age for a typical single household coop to ~1000 birds of multiple ages for the group facility. Thirteen households in 7 villages were engaged in rearing production birds. In each coop we sampled five production birds, or five birds of each age group (in weeks) if more than one batch was being raised concurrently.

'Household birds' included varietals not intended for commercial sale that are not held in coops, eat scraps and ground maize rather than formula feed, and do not receive antibiotics. We sampled 5-10 household birds from each village, regardless of whether the villagers were actively engaged in production poultry farming.

For all birds, we collected sterile cloacal samples using swabs that were placed in Cary Blair transport medium (Becton Dickinson, Franklin Lakes, NJ) and streaked directly on McConkey-Lactose agar for isolation.

Environmental samples

Samples from household drinking water, soil from house surroundings, and kitchen surfaces were collected from households associated with production or household bird samples, in order to characterize the domestic environment. In addition, soil and surface samples were collected from coops in order to characterize the production environment.

Household water

During the first half of the study period, spanning the period between August 2010 and January 2012, we collected 50mL water samples from two different household storage containers in Whirl-Pak bags (NASCO Corp., Fort Atkinson, WI) in the same manner that water was dispensed for drinking. If there was only one drinking water container available, then the second sample was taken from water used to wash dishes or bathe. For the remainder of the study period, only one sample per household was obtained from drinking containers in order to reduce sampling effort, as there were no notable differences in the *E. coli* recovered across repeated samples.

Samples were processed using membrane filtration for isolation of *E. coli*. Two volumes varying between 3-50mL of water were filtered depending on the expected concentrations of *E. coli* for a particular water source, based on prior field testing. Because the goal was to recover and isolate *E. coli*, it was not necessary to keep the quantities uniform. Membranes were plated onto Chromocult agar (Merck, Darmstadt, Germany) and incubated at ambient temperature for 48 hours.

Soil

During the first half of the study period, we collected two samples of approximately 15cm^3 from around the house and from around the poultry coop, if one was present. Soil

from just below the surface was placed into a conical tube using a sterile plastic spoon that was discarded after use. Samples were stored on ice until processing in the lab, within 4-6 hours. For the second part of the study period, only a single sample was obtained from the household yard, due to the rarity of coops and difficulty in recovering *E. coli* from these samples.

Each soil sample was diluted with 30mL of deionized water and mixed thoroughly to obtain a 2:1 dilution. A second dilution was made with an additional 10mL of deionized water. The supernatant of the original sample and the dilution were streaked separately onto Chromocult agar for isolation.

Surfaces

During the first half of the study period, we collected household surface samples from two locations: where food was prepared (e.g., a cutting board) and where food was eaten (e.g., a table). A 28x30cm plastic stencil was used to define a consistent sampling area. If the area was dry, we used a cotton tip applicator that had been submerged in 1mL 0.9% NaCl solution to obtain the sample. If the area was wet, a dry swab was used and placed in Cary Blair transport medium (Becton Dickinson, Franklin Lakes, NJ). Two surface samples from the inside or outside of the coop, usually constructed from cement or wood, were also taken using the same procedures. Surface samples were plated directly onto Chromocult agar and streaked for isolation. For the second part of the study period, surface samples were not collected due to the low rate of isolate recovery.

Sample processing

All plates were incubated at ambient temperature, ranging from 32-35^{o}C. After approximately 24-48 hours, up to four 4-methylumbelliferyl-beta-D-glucuronide (MUG) -

positive *E. coli* colonies from each sample isolated on Chromocult agar were randomly selected and transferred onto MacConkey Lactose (MKL) agar to confirm the presence of *E. coli*. If *E. coli* was not initially isolated upon first plating on Chromocult agar due to its low abundance or contamination with other bacteria, efforts were made to re-isolate colonies on Chromocult agar before transferring onto MKL agar. After a 24-hour incubation at 37°C, lactose-positive (lac+) *E. coli* colonies were transferred from MKL back onto Chromocult to ensure pure isolates, and incubated for another 24 hours.

We randomly selected up to four *E. coli* colonies from soil and fecal samples and two from water and surface samples to test for AR. More isolates from soil samples were used than from water or surfaces because we expected higher microbial diversity in soil. The selected colonies were streaked onto nutrient agar slants and incubated for 24 hours at 37°C. Bacteria from these slants were then placed into a 0.9% NaCl solution to obtain a turbidity matching a 0.5% McFarland Standard. Using this solution, bacteria were plated onto Mueller-Hinton Agar, creating a lawn for resistance testing.

Antibiotic sensitivity was assessed using the Kirby-Bauer disc diffusion method (Bauer et al. 1966), with 12 antibiotics: ampicillin, amoxicillin/clavulanate, cefotaxime, cephalothin, chloramphenicol, ciprofloxacin, enrofloxacin, gentamicin, streptomycin, sulfisoxazole, trimethoprim/sulfamethoxazole and tetracycline (Becton Dickinson, Franklin Lakes, NJ). Beta-lactams (ampicillin, amoxicillin/clavulanate, cefotaxime, cephalothin), fluoqoruinolones (ciprofloxacin, enrofloxacin) trimethoprim/sulfamethoxazole and sulfisoxazole are commonly used as first- or second-line agents to treat *E. coli* infections in humans, and tetracycline, chloramphenicol and sulfonamides are frequently used as

additives in animal feed. Zones of inhibition were measured after a 24-hour incubation period.

Statistical analysis

Our main outcome of interest was the zone of inhibition around each of the tested antimicrobial discs. A secondary outcome was the categorical interpretation of the zone (susceptible or non-susceptible) based on our custom breakpoints.

Most studies of AR construct antibiograms using categorical interpretations (susceptible, resistant) of minimum inhibitory concentrations or corresponding disk diffusion zone of inhibition. The categorization is based on externally defined consensus breakpoints set by organizations such as the US Clinical Laboratory Standards Institute (CLSI) or its European equivalent EUCAST. Using the distributions of zones of inhibition of each antibiotic for all isolates in our data, we derived custom susceptibility breakpoints that may more accurately reflect the local population of bacterial strains compared to externally defined breakpoints based on wild-type cutoff values (Wiggins 1996). These custom breakpoints might have limited clinical utility, but are more consistent with the objective of our study to describe the local ecological dynamics of *E. coli* resistance phenotypes.

To define susceptibility breakpoints, we fitted two-component mixture models to zone diameter distributions of production bird isolates to index isolates into susceptible and resistant populations. We selected among three expectation maximum algorithms from R's mixtools package(Benaglia et al. 2009): a parametric, semi-parametric and non-parametric specification that accounted for repeated measurement data to account for multiple isolates cultured from each sample. Among those, the best-fit model was selected based on the log-likelihood statistic. Custom breakpoints were set where the density estimates for the fitted distributions intersect, as described by Budczies et al., (2012). These

updated breakpoints were rounded to the nearest whole number and used to categorize all isolates in our data as susceptible and resistant.

We used differences in the modality of zone distributions to identify patterns that distinguished phenotypic resistant patterns associated with particular sources of isolates, e.g., production versus household birds or coop versus household surfaces. Distributions were considered unimodal, suggesting a population of all resistant or all susceptible bacteria, if Hartigans' dip statistic (which measures multimodality in a sample) had a *P*-value >0.1, failing to reject the null hypothesis of unimodality (Hartigan and Hartigan 1985), or if the estimated proportion of a component in a mixture was lower than 0.01 (Díaz-Muñoz et al. 2013). Otherwise, the distributions were considered bimodal suggesting a mixture of both susceptible and resistant bacterial populations.

We compared the kernel density estimates of zone distributions and the percentages of non-susceptible isolates across categories of interest (type and source of sample). To account for the hierarchical, unbalanced nature of the data when comparing across categories, we used generalized linear mixed models (GLMM) to model the outcome of AR. An identity link function was used for continuous outcome measures (inhibition zone size; GLMM-ANOVA), and a binomial one for the categorical interpretation of the zones (susceptible and non-susceptible; GLMM-logit). In all specifications, we used nested random effects with varying intercepts and slopes by sample, household and village levels to capture unobserved heterogeneity between the multiple levels and account for repeated sampling.

In addition to examining resistance to each drug, the resistance profile for each isolate was used to perform hierarchical cluster analysis. We normalized the zones of inhibition, computed Euclidean distances between each isolate, and ran a hierarchical clustering algorithm with an agglomeration method based on the average distance between

categories, implemented in R's flashClust package(Langfelder and Horvath 2012). Support values were calculated via multiscale bootstrap resampling with n=1000 replications.

All analyses were carried out in R version 3.0.1 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Quantitative characterization of poultry production

Monthly census surveys show wide variability in the number of production birds being raised over time. Fifteen villages where data were available were categorized based on the maximum number of birds recorded by our surveillance surveys during the study period (high intensity: >500 birds, *n*=4; medium intensity: 150-500 birds, *n*=7; low intensity: <150 birds, *n*=4; no data available, *n*=2). In all villages production was intermittent, with periods when no broilers were raised. At the time of sampling visits, birds were actively farmed in 7 villages.

Qualitative characterization of poultry production

Ethnographic interviews also confirmed that poultry production varies widely both over time and in scale: while some households periodically maintain flocks of ~10 birds, others consistently house hundreds. Most backyard coops are located within 50m of houses in raised open structures or directly below the family home. Two villages in the region had large facilities (for housing up to 1500 birds of multiple ages) built with foreign and local government aid, located away from other houses, and run cooperatively by a group of community members.

Ethnography also confirms that poultry production in this region is highly intermittent and characterized by "boom and bust" cycles. Development projects commonly provide training, birds and initial supplies. However, once outside support is withdrawn, production may become unsustainable due to fluctuating demand, outbreaks of diseases and other factors negatively impacting yield, and lack of reinvestment in the flock. This is

particularly true in remote communities where resale is often difficult, or when markets are episodic (e.g., for holidays) or seasonal.

Villagers state convenience as a key reason for engaging in farming: the production cycle is highly integrated, with a single firm providing hatchery-raised animals and branded feed. Feed and additional supplies (antibiotic supplements and vaccines) are acquired from local veterinary pharmacies or development agency offices. Little training is required to engage in production, as suppliers provide detailed manuals with guidelines.

Most farmers raised broiler chickens for meat production, with fewer households raising laying hens. The dominant broiler breed reached a maximum weight of 3.5-4 kg within 8 weeks, but birds were usually slaughtered around week 6 as decreasing marginal weight gain after that point makes longer cycles less profitable.

Poultry feed came from three local manufacturers (Pronaca, Agripac, and Nutril). Corn was the principal ingredient in all varieties, with additional protein from seafood added to accelerate growth. The ingredients of the feed listed unspecified "antibiotics." Guidelines from suppliers and veterinarians instructed villagers to add additional antibiotic and vitamin supplements to the animals' water on certain days. For example, Nutril's guidelines suggest application of unspecified "vitamins" on days 1-3, 8, 16-17, 22, 25, and "antibiotics" on days 9-10 and 23-24, as well as when a respiratory infection was noted. Several of the packets branded as " vitamins" for sale by veterinary supply stores had antibiotics listed in the ingredients (in particular, oxytetracycline, streptomycin, and enrofloxacin), suggesting that administration of vitamins may also have introduced these antibiotics to the animals, even in the absence of additional antibiotic administration. There was no evidence that villagers were instructed in the practice of ceasing supplementation with antibiotics one week prior to slaughter. Villagers cited a wide variety of practices with

respect to application of these supplements, with some applying more than the recommendations called for and others not supplementing their flock at all, usually due to financial constraints.

In contrast to production birds, household birds were hatched in villages and raised for local consumption. These birds are markedly different in appearance and better suited to local conditions. Interviews with local farmers and veterinary store owners confirmed that these birds were rarely if ever given antibiotics. They were fed corn, yucca, coconut, and scraps of food instead of commercial feed.

Chemical analysis of commercial poultry feed

Analysis by mass spectrometry detected antibiotics in all types of Nutril-brand feed that were sampled. Feed intended for broilers and laying hens contained virginiamycin, chloramphenicol and lincomycin. Tetracycline was found in plain ground corn not formulated specifically as poultry feed (). Our surveys determined that the majority of birds (75% of 81 flocks surveyed) were given Nutril-brand feeds. Two of the four antibiotics added to the feed (tetracycline and chloramphenicol) are active against *E. coli* and were included in our testing panel.

Sample characterization

Counts of isolates and samples positive for *E. coli* included in the analysis are shown in Table 1. Household birds (*N*=360) were sampled from 226 households in all 17 villages, yielding 1089 isolates. Production birds (*N*=262) were actively being raised at the time of sampling in 35 households in 10 villages, yielding 786 isolates. In seven of the villages, sampling visits coincided with periods when there were no flocks in production, either because nobody in the community was engaged in poultry farming during the entire

surveillance period (2 villages), or because no community member had active flocks at the time of the visit (5 villages); we only collected samples of household birds from these villages.

Environmental samples were collected from 190 households in 17 villages. *E. coli* was recovered in 114 and 187 of household water and soil samples, respectively. In the first 98 houses that were visited we collected household surface samples, and were able to recover *E. coli* from 54 samples. Coop surface and soil samples were also collected if there was a coop present on site (with or without active farming), and were positive in 32 and 34 cases, yielding 77 and 96 isolates, respectively. In the second half of the study, including 9 of 17 villages, the protocol was modified and sampling of these sites ceased due to low recovery rates.

Table 1: Counts of *E. coli* isolates, by sample type collected from 17 villages in Esmeraldas Province, Ecuador, 2010-2013. Production birds include broilers and laying hens.

Sample type	# of *E. coli* Isolates	# of Samples	# of Households	# of Villages
POULTRY	1875	622	226	17
Production	786	262	35	10
Household	1089	360	206	17
ENVIRONMENT	1460	529	190	17
Household water	326	144	114	17
Household soil	863	265	187	17
Coop soil	96	34	17	6
Household surfaces	98	54	46	8
Coop surface	77	32	14	5

Custom susceptibility breakpoints

Table 3 shows the official resistant and susceptible breakpoints defined by CLSI, and the custom values estimated using our approach and subsequently applied in the analysis, as well as the percentage of isolates that would be classified as resistant under each scenario. Figures showing the fitted mixture models are available in . For all drugs, the custom breakpoint was lower than the official susceptible one, but for most drugs the custom breakpoints were in the officially defined intermediate range. While for most drugs the use of custom breakpoints in place of official ones would change <10% of classifications, for amoxicillin/clavulanate, cephalothin, streptomycin and enrofloxacin the use of custom breakpoints resulted in a >30% difference in how isolates were classified. Use of the CLSI

breakpoints would have resulted in classifying isolates that were more likely to belong to the susceptible population as resistant. This point is well illustrated by comparing results for enrofloxacin and ciprofloxacin: the zones of inhibition for these very similar antibiotics had a Person correlation of 0.951, and one would expect the proportion of isolates resistant to each to be similar. The use of CLSI breakpoints would have classified 24% of isolates as enrofloxacin-resistant and 12.4% as ciprofloxacin-resistant, while our custom breakpoints yield frequencies of 10.9% and 11%, respectively.

Table 2: Official and custom susceptibility breakpoints. Custom breakpoints used to derive categorical resistance profiles for all *N*=3860 isolates used in the analysis.

Drug	CLSI breakpoint range, Resistant–Susceptible, mm	Custom breakpoint, mm	% Non-susceptible, CLSI susceptible BPs (N=3860 isolates)	% Non-susceptible, custom BPs (N=3860 isolates)
Amoxicillin/clavulanate	13–18	14.62	12.77%	7.89%
Ampicillin	13–17	12.38	28.22%	23.06%
Cefotaxime	22–26	25.59	10.22%	10.19%
Cephalothin	14–18	9.4	60.84%	8.34%
Chloramphenicol	12–18	15.8	12.41%	11.90%
Ciprofloxacin	15–21	18.2	12.44%	11.03%
Enrofloxacin	16–23	15.35	24.11%	10.94%
Gentamicin	12–15	13.17	6.06%	5.40%
Streptomycin	11–15	8.51	39.43%	14.06%
Sulfisoxazole	12–17	12.83	34.36%	32.83%
Tetracycline	11–15	14.33	43.33%	43.30%
Trimethoprim/sulfamethoxazole	10–16	13.86	29.78%	29.57%

Note: Custom breakpoints were defined using two-component mixture models to zone distributions using parametric, semi-parametric and non-parametric expectation maximum algorithms for repeated measurement data to account for multiple isolates cultured from each sample. The best-fit model was selected based on the log-likelihood statistic. Custom breakpoints were set where the density estimates for the fitted distributions intersect. These updated breakpoints were rounded to the nearest whole number and used to categorize all isolates in our data as susceptible and resistant. CLSI breakpoints based on accepted veterinary breakpoints (Clinical and Laboratory Standards Institute (CLSI) 2009), or clinical ones for drugs not approved for veterinary use (Clinical and Laboratory Standards Institute (CLSI) 2012)

Antibiotic resistance in poultry samples

Production vs household poultry

Production birds, including broilers and laying hens, had a notably higher proportion of resistant isolates than household birds. Seventy eight percent of production birds and 34% of household ones were resistant to tetracycline. More than half of production bird isolates were resistant to sulfisoxazole and trimethoprim/sulfamethoxazole (69% and 63%, respectively), as compared to 20% and 17%, respectively, in household birds (Table 2). The lowest resistance was to gentamicin (16% of production and 1% of household bird isolates) and amoxicillin/clavulanate (18% and 2%). The difference between production and household bird isolates was significant ($P<0.01$) for all drugs by GLMM-logit, also reflected by significantly lower zones of inhibition ($P<0.01$ for all drugs by GLMM-ANOVA) (Figure 1). When the CLSI breakpoints (shown as dotted line in Figure 1) were used in place of custom ones (dashed line), the results were similar with the exception of a non-significant difference for cephalothin ($P=0.56$) where 60% and 63% of production and household isolates would have been classified as resistant.

Examining the differences in the modality of zone distributions, we observed a phenotypic pattern or resistance unique to production birds. Distributions for amoxicillin/clavulanate, cephalothin, gentamicin and streptomycin, shown in the top row of Figure 1, showed bimodal tendencies for production birds, suggesting a mixed population of susceptible and resistant strains, and unimodal tendencies for household birds, suggesting the presence of strains susceptible to these drugs. The estimated proportion of the resistant population in the best-fit mixture model was <0.01 for these four drugs; $P>0.1$ by Hartigan's Dip Test for all but cefotaxime. We refer to this particular phenotypic pattern

hereafter as a 'production bird signature,' as this pattern suggests the presence of resistant phenotypes unique to production birds not found in household birds. In contrast, distributions for all other drugs were bimodal for both types of poultry, suggesting that the same resistance phenotype is present in both samples, although for all drugs the resistance phenotype was more prevalent in production birds.

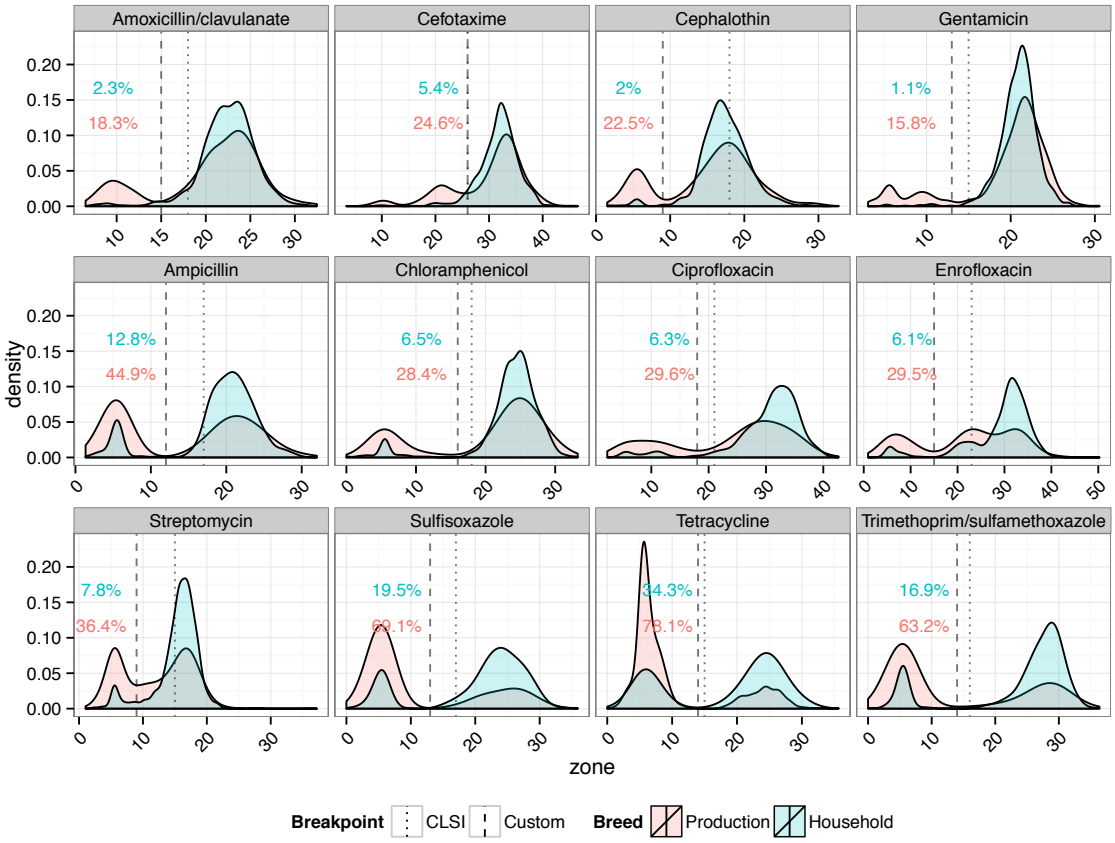
Table 3: Percentage of *E. coli* isolates resistant to a panel of 12 antibiotics, by sample type

	AMC	AM	CTX	CF	C	CIP	ENO	GM	S	G	TE	SXT
POULTRY	9.01	26.24	13.44	10.61	15.68	16.11	15.89	7.25	19.79	40.27	52.64	36.32
Production	18.32	44.91	24.55	22.52	28.37	29.64	29.52	15.78	36.39	69.08	78.12	63.23
Household	2.30	12.76	5.42	2.02	6.52	6.34	6.06	1.10	7.81	19.47	34.25	16.90
ENVIRONMENT	6.44	18.97	6.03	5.41	7.05	4.52	4.59	3.01	6.71	23.29	31.30	20.89
Household water	12.27	26.38	4.60	10.12	6.13	4.29	4.60	2.15	4.60	26.07	29.14	23.01
Household soil	4.17	15.30	5.45	2.32	6.14	3.36	3.36	2.67	4.98	18.19	27.46	15.87
Coop soil	4.17	17.71	8.33	8.33	9.38	7.29	7.29	5.21	12.50	35.42	53.13	35.42
Household surfaces	2.04	20.41	3.06	2.04	6.12	1.02	1.02	0.00	9.18	24.49	28.57	19.39
Coop surface	15.58	28.57	19.48	20.78	19.48	19.48	19.48	11.69	24.68	51.95	59.74	51.95

Note: numbers show percentage of isolates classified as resistant based on their zone of inhibition. Categorical interpretation is based on breakpoints derived as described in the Methods section. The number of isolates tested for each sample type is shown in Table 1. AMC – amoxicillin/clavulanate, AM – ampicillin, CTX– cefotaxime, CF – cephalothin, C – chloramphenicol, CIP – ciprofloxacin, ENO – enrofloxacin, GM – gentamicin, S – streptomycin, G – sulfisoxazole, TE – tetracycline, SXT – trimethoprim/sulfamethoxazole.

Figure 1: Kernel density estimates of inhibition zones and categorical interpretation of susceptibility tests of *E. coli* isolates from production (red) and household (blue) bird samples;

overlapping portions colored in grey. Production birds include broilers and laying hens. Percentage of resistant isolates in each sample are shown in corresponding colors. Dashed lines show the custom susceptibility breakpoint derived from using a mixture model and used to derive that categorical interpretation. Dotted lines show the consensus clinical breakpoints used by CLSI (Clinical and Laboratory Standards Institute (CLSI) 2010). For production birds *N*=786 isolates from 262 birds; for householdbirds *N*=1089 isolates from 360 birds.

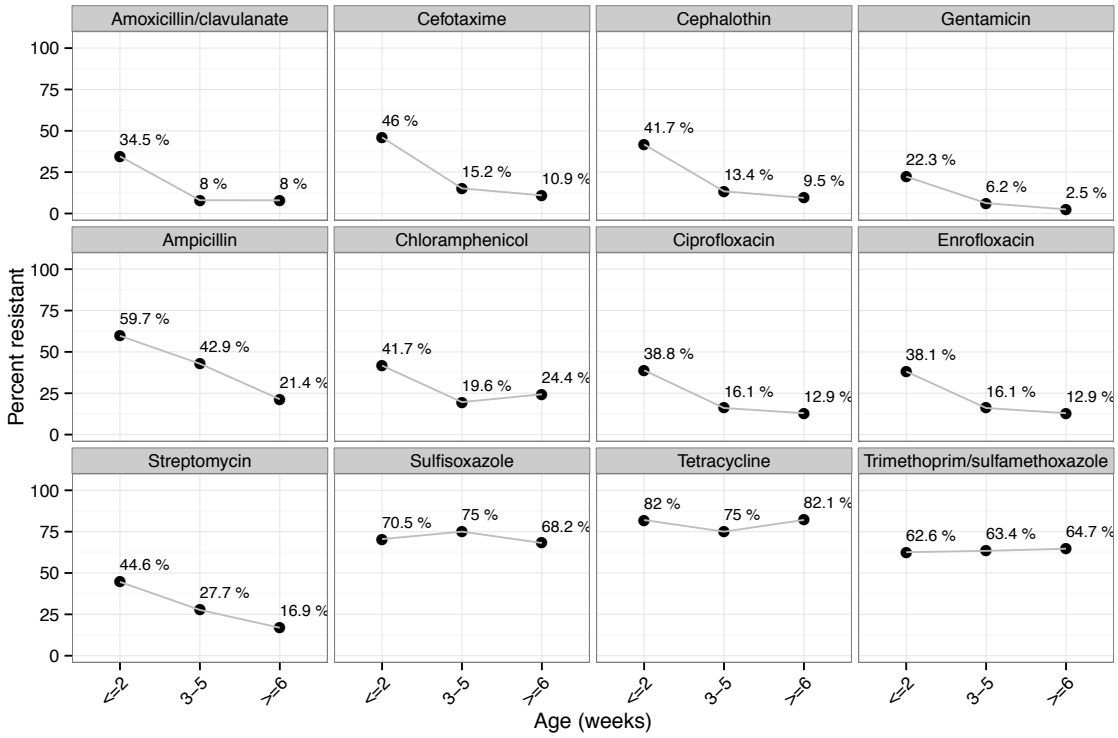


Production birds by age

The prevalence of resistant phenotypes tended to decrease with bird age (Figure 2) for all drugs ($P<0.05$ by GLMM-ANOVA) except those with the highest resistance levels (i.e., sulfisoxazole, trimethoprim/sulfamethoxazole and tetracycline). This suggests that production birds arrive pre-colonized with strains resistant to some drugs, likely acquired outside the study system, and this carriage declines with age. Age was not available for household birds. However, the oldest group of production birds (>6 weeks) still had resistance levels that were significantly higher than those among household ones ($P<0.01$ by GLMM-ANOVA and GLMM-logit). When using CLSI breakpoints, the decline in resistance from youngest to middle age groups was of similar significance. However, there was an uptick from the middle to the oldest age group in resistance to cephalothin, enrofloxacin, ciprofloxacin and streptomycin.

Figure 2: Categorical resistance of *E. coli* isolates from production birds (broilers and laying hens), by age of bird

Dots show the frequency of resistant isolates for each age group. Generalized linear mixed effects model of the zone of inhibition regressed against age, with bird included as a random effect, showed a significant decline in resistance (P<0.05) for all drugs with the exception of sulfisoxazole (P=0.82), trimethoprim/sulfamethoxazole (P=0.9) and tetracycline (P=0.47). N=452 isolates from 164 birds.

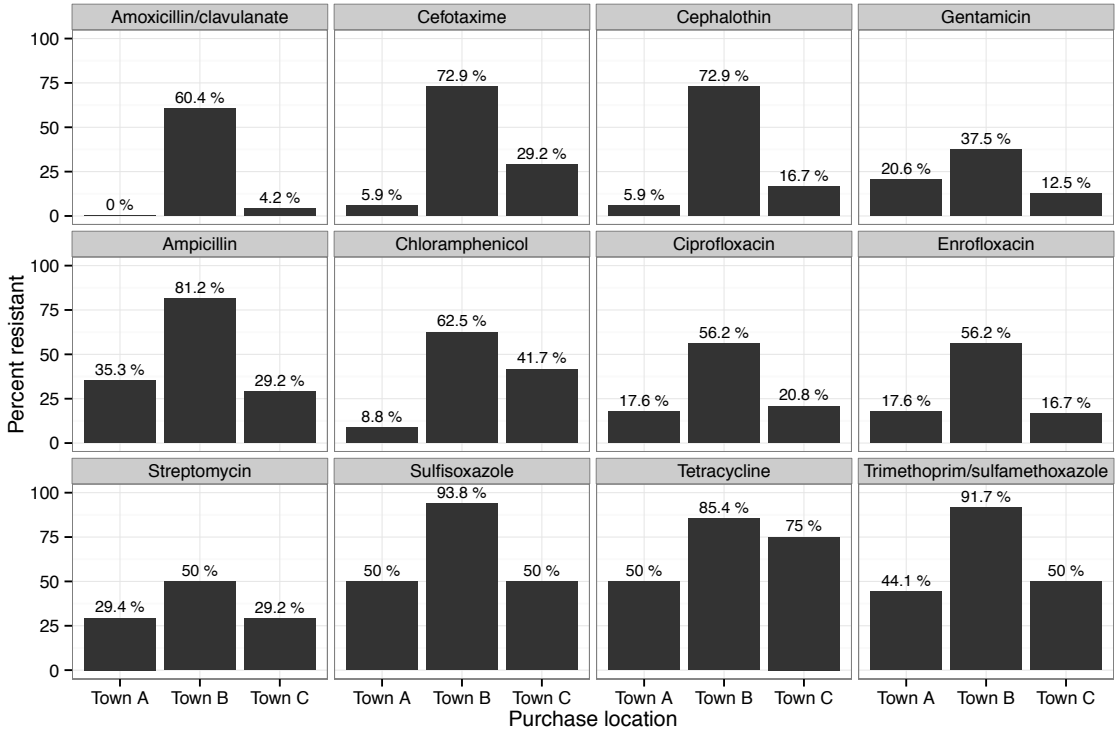


Production birds by point of purchase

The prevalence of resistance among production birds <2 weeks differed by the location where chicks were purchased (Figure 3) for all drugs (P<0.01 by GLMM-logit), with the exception of tetracycline (P=0.08). Resistance, particularly to the production bird signature drugs (shown in the top row of Figure 3), was always highest for chicks purchased in Town B, a large city outside the immediate study system. Results would be similar in terms of significance if CSLI breakpoints had been used, with the exception that the frequency of resistance to cephalothin in Town B would not differ significantly from that in Town C.

Figure 3: Antibiotic resistance in *E. coli* isolates from production birds (broilers and laying hens) of age <2 weeks by purchase location

Location based on survey conducted at time of sample collection. Town A (*N*=34 isolates from 12 samples) and Town C (*N*=24 isolates from 9 samples) were significantly different (*P* <0.05) from Town B (*N*=48 isolates from 17 samples) by GLMM-ANOVA and GLMM-logit. (*N*=106 isolates from 38 samples)



Production birds with added antibiotics in water

Detailed surveys on poultry rearing practices were available for 122 birds in 27 households, including a question about antibiotic administration to the flock's water supply, in addition to antibiotics provided in the commercial feed. Farmers reported adding penicillin+streptomycin, tetracycline, sulfonamide, sulfametazine+trimethoprim, piperacillin, erythromycin, sulbactam, and/or enrofloxacin. Out of 20 farming households surveyed in detail about poultry raising practice, 16 (80%) reported supplementation with antibiotics. No significant differences were observed between birds with and without

reported supplemental antibiotic administration (P >0.05 for all drugs by GLMM-ANOVA and GLMM-logit).

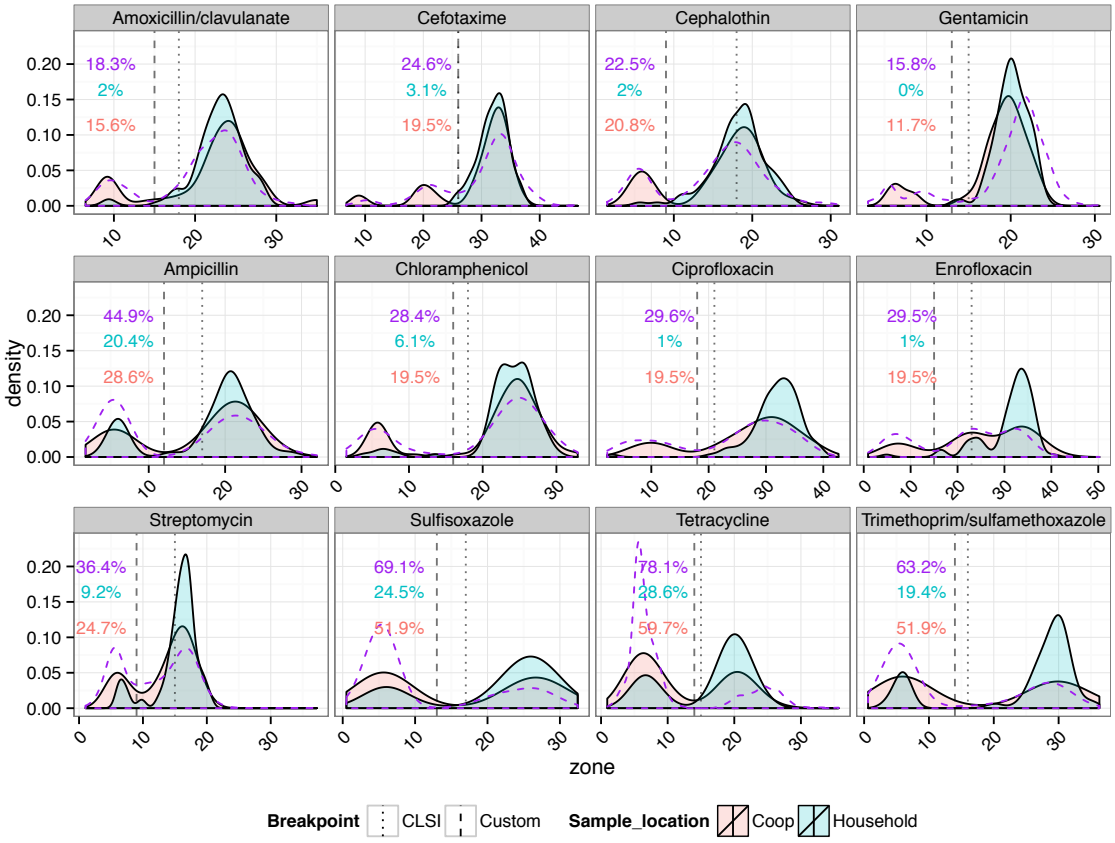
Antibiotic resistance in environmental samples

Soil and surface isolates from coops and households

Resistance patterns in coops were different from households, but very similar to those in production birds. Surface isolates from coops showed higher resistance levels than surface isolates from the domestic environment (P<0.05 by GLMM-logit and GLMM-ANOVA with the exception of ampicillin and chloramphenicol) (Figure 4). In addition, surface isolates from coops also exhibited the production bird signature pattern of resistance (shown in the top row of Figure 4).

Figure 4: Kernel density estimates of inhibition zone profiles and categorical interpretation of susceptibility tests for *E. coli* isolates from household and coop surfaces, and production birds (broilers and laying hens)

Kernel density estimates for the distributions of coop (red), household (blue) and production bird (dashed purple) isolates, with the overlapping portions between coop and household colored in grey. Percentage of resistant isolates in each sample are shown in corresponding colors. Dashed vertical lines show the custom susceptibility breakpoint derived from using a mixture model and used to derive that categorical interpretation. Dotted lines show the consensus clinical breakpoints used by CLSI (Clinical and Laboratory Standards Institute (CLSI) 2010). For coops *N*=77 isolates from 32 samples; for households *N*=90 isolates from 54 samples; for production birds *N*=901 isolates from 300 birds.



Water and soil isolates by farming history of household

Using household survey data on past experiences with production poultry farming, we divided households into those that had farmed production birds within the past year,

had farmed production birds over one year before the sampling event, and those that had never farmed production birds.

For household water samples, inhibition zone profiles and their categorical interpretations showed no significant differences across groups (P>0.05 by GLMM-ANOVA,). For household soil samples, the only significant differences were for amoxicillin/clavulanate (P<0.01 by GLMM-ANOVA), ampicillin (P =0.04), cephalothin (P<0.01), sulfisoxazole (P <0.01) and trimethoprim/sulfamethoxazole (P = 0.03), and households that had never farmed poultry actually had a slightly higher proportion of resistant isolates ().

Water and soil isolates by farming history of village

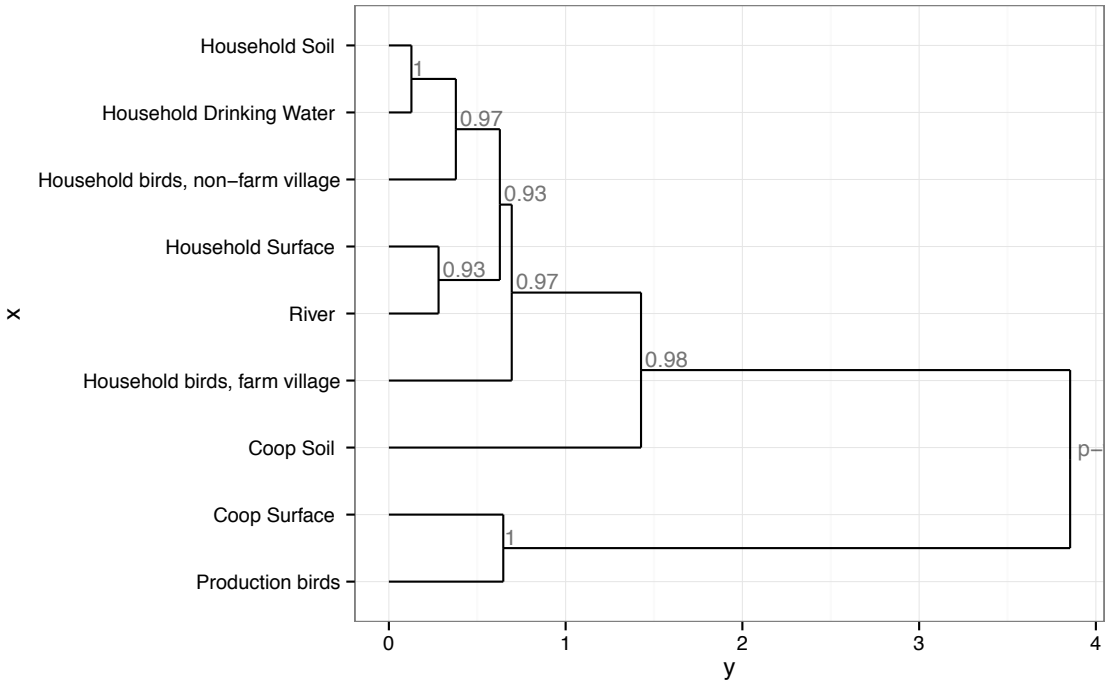
We were also interested in whether poultry farming had an effect on resistance profiles at a larger scale. Using the monthly survey data on poultry production, villages were categorized into high, medium and low-intensity farming, respectively defined as >400 broilers, 100-400 and <100 broilers raised in the one year prior to environmental sampling. A comparison of resistance profiles showed no difference between the three categories for household water samples, with the exception of ampicillin (P=0.008 by GLMM-logit) and sulfisoxazole (P = 0.01), where samples from villages without poultry farming in the past year had higher frequency of resistant isolates (). The same comparison for soil samples showed no difference between groups, with the exception of amoxicillin/clavulanate (P =0.03 by GLMM-logit), gentamicin (P < 0.01) and tetracycline (P < 0.01), where samples from villages with mid-intensity poultry farming in the past year tended to be less resistant than those from high- or low-intensity villages ().

<u>Cluster analysis of resistance patterns across all sample sources</u>

To visualize the relative similarity of AR patterns across all sample types, we performed hierarchical clustering analysis using the normalized zones of inhibition. The dendrogram, including bootstrap support values (Figure 5), shows that production bird and coop surface isolates form a distinct clade from householdbirds, water and soil isolates. Coop soils and cloacal isolates from household birds raised in villages with active farming are closest to the production bird phenotypic pattern.

Figure 5: **Hierarchical clustering by sample type and location**

Based on resistance profiles of *N* =3860 *E. coli* isolates of environmental and poultry samples. Numbers show *p*-values from multiscale bootstrap resampling with *n*=1000 replications



DISCUSSION

In this study we used an ecological lens to examine environmental and animal reservoirs of AR in the context of small-scale poultry farming. We found that: 1) High levels of AR were found in both the production and household environment, although production birds and coops showed substantially higher levels of resistance than household birds and domestic environmental samples. 2) Production birds exhibited a distinct phenotypic pattern from household birds. The phenotypic signature associated with production birds was more prevalent among younger animals and was associated with a particular source of chicks, suggesting the introduction of AR from outside sources; 3) Surfaces of poultry coops showed a phenotypic profile distinct from household soil and water samples and similar to that of production birds, underscoring the potential of poultry production to serve as a source of resistant strains for villagers through occupational exposure. However, poultry farming status did not have an effect on AR in isolates from water or soil, at the household or community level.

In *E. coli* isolates that we recovered from production birds we found very high levels of resistance to drugs used in human medicine, particularly tetracycline, trimethoprim/sulfamethoxazole and sulfamethoxazole, to which over half of isolates were resistant. Rates of resistance to broad-spectrum antibiotics amoxicillin/clavulanate (15%) and fluoroquinolones (30%) was substantially higher than that found via abattoir surveillance carried out on industrial farms in United States (9% and <1%, respectively) (USDA 2011) and in Canada (8% to nalidixic acid) (Public Health Agency of Canada 2012). Even in household environment we found relatively high levels of resistance to broad-spectrum antibiotics, particularly in drinking water where the proportion of isolates resistant to amoxicillin/clavulanate, ampicillin and cephalothin was higher than that in household birds.

In line with studies from other regions in the world, AR in intensively farmed animals tends to be higher than in free-range or organically-raised varieties (van den Bogaard et al. 2001; Obeng et al. 2012; YOUNG et al. 2009; Walk et al. 2007). For example, a study of 20 poultry flocks with over 500 birds from Germany found resistance rates and mean minimum inhibitory concentrations of bacteria isolated from organic keeping systems had lower values than those from conventional ones, especially for *E. coli*. Free-range Tibetan pigs (*N*=232) had lower levels of resistance among *E. coli* and Enterococci than intensively raised pigs from other parts of China, particularly to tetracycline and other antibiotics known to be used in farming (Li et al. 2014).

The higher prevalence of resistance in intensively-farmed versus conventionally-raised animals in similar studies is explained by the administration of systemic antibiotics in feed and water (Diarra et al. 2007; Avrain et al. 2003). However, our study was observational and we were unable to vary this exposure. From the survey data we have, we did not detect differences between birds reportedly receiving antibiotic supplements to the diet, in addition to what is already present in commercially mixed feed. While this counters the results of other studies (Diarra et al. 2007; Bonnet et al. 2009a), the exposure in our data was based on self-reporting and therefore subject to recall and/or misclassification bias. We find more evidence in support of an alternative explanation that production chickens are colonized with resistant strains in the hatcheries prior to their arrival in village coops.

Resistance to amoxicillin/clavulanate, cefotaxime, cephalothin and gentamicin was particular to production bird isolates, as determined by a mixture model analysis of the zones of distribution. A genotypic analysis of human isolates from the same study system (Moser et al. 2015) points to a molecular basis for these phenotypic groupings. Resistance

to these drugs in human isolates was characterized by a synergistic interaction between production bird exposure and the presence of the int1 gene, a marker for the carriage of mobile genetic elements that can house a wide range of linked resistance genes at a low fitness cost (Rowe-Magnus, Guerout, and Mazel 2002).

Resistance to the four "signature" drugs identified above, and to fluoroquinolones, streptomycin, chloramphenicol and ampicillin, decreased with animal age and was higher among birds sharing the same origin. The decline of resistance with production bird age suggests that farmed poultry start their growth cycle pre-colonized with antibiotic resistant bacteria, rather than acquiring resistance in the village environment as a result of any particular farming practice. This pattern has been previously reported in the literature. A study of 293 *E. coli* isolated from French laying hens, which reported higher carriage in younger birds of $bla_{CTX\text{-}M}$ genes that confer resistance to most beta-lactam antibiotics (Chauvin et al. 2013). A large case-control study in Canada of 197 isolates from broilers sampled longitudinally and exposed to different antibiotic regimens also found an overall decrease in the prevalence of resistance between days 7 and 35 in both cases and controls, and a concurrent decrease in carriage of *int1* and *tet* genes (Diarra et al. 2007).

While it is possible that the decline in resistance prevalence is part of the normal course of *E. coli* community dynamics or changes in the host's physiology, our hypothesis that this production bird signature is imported from outside the study villages is further supported by the result that chicks purchased from a veterinary store in one particular town – a large regional center – were several times more likely to be resistant to all drugs, especially the ones with the production bird signature. Our ethnographic interviews with a veterinary store owner in this town revealed that he regularly supplemented the chicks'

drinking water with antibiotics as soon as they arrived at his shop, and before re-sale to customers.

We found that frequently touched surfaces of poultry coops had resistance profiles most similar to those of production birds, likely due to environmental proximity and transmission via the hands of farm workers. Drinking water and backyard soils exhibited resistance patterns more similar to those of household birds.

We did not detect a difference in AR profiles between water samples from farming versus non-farming households, and we observed no indication that villages with higher farming intensity had higher rates of AR. The lack of observed effect of poultry production on the domestic environment may have to do with the large monthly variation in the number of raised production birds within and across villages. It is possible that the intermittent nature of intensive production does not sustain sufficient pressure to affect bacterial populations within the household environment. However, it is also possible that the frequency of sampling and culturing methods we used did not offer enough power to detect the effect of added systemic antibiotics that other studies have reported (Avrain et al. 2003; da Costa et al. 2011; van den Bogaard et al. 2001; Diarra et al. 2007)

Ascertaining the role of poultry farming in the broader environmental transmission and the community ecology of AMR at a landscape (village) level will require additional sampling and a metagenomic approach. However, the high rates of AR we observed on the surfaces of poultry production facilities in these villages may present a more localized occupational risk for acquisition of AR by poultry farmers, and has the potential to impact humans living in the communities through contact with farmers as well as poultry consumption. In a related study in the villages under study here, we found that human fecal samples from poultry farmers exhibited higher levels of phenotypic and genotypic (class-1

integron) resistance, with prevalence of *int1* among farmers of production birds over twice as high as those that raised household ones (Moser et al. 2015). Thus, small-scale farming may present occupational risk to farmers and villages in our study system, similar to what has been found in larger-scale industrial operations (van den Bogaard et al. 2001; Alali, Scott, and Norby 2010; Neyra et al. 2014). The idea that farming is an occupational risk for AR is supported by literature dating back to the 1970s (S. B. Levy, FitzGerald, and Macone 1976), see review (Marshall and Levy 2011b).

Study limitations and avenues for future research

This study was limited by several factors. First, our study relies on variations in the use of antibiotics and the intensity of poultry production inherent to the local context of the study region that are difficult to characterize and control for. Because of the nature of our visits to the villages, we were unable to exploit the wide variability in animal density over time to look more closely at the correlation between temporal variability and resistance patterns. Second, our analysis relies on phenotypic resistance data, and therefore we did not have the opportunity to account for the multiple genetic determinants and expression patterns that underlie resistance. Third, we did not test for resistance to two of the three antibiotics that our chemical analysis found in the poultry feed – virginiamycin and lincomycin, both narrow-spectrum antibiotics active against Gram-positive bacteria and not *E. coli.* Future studies should culture *Enterococcus* spp. as an additional sentinel species to capture potential effect of antibitoci selection on Gram-positive organisms, as past research has suggested effects differ for bacterial species(da Costa et al. 2009). Fourth, we use *E. coli* as a sentinel organism and rely on culture-based methods, capturing only a fraction of the complex, multilevel interactions between environment, host, microorganism and horizontally-transferred genetic elements. Future similar studies could use a metagenomic

approach to characterize the diversity of environmental and animal reservoirs, as well as the patterns and mechanisms of resistance gene exchange between these bacterial communities (Forsberg et al. 2012).

Despite these limitations, our study provides a large dataset to characterize prevalence of resistance among animals and household environments in the context of small-scale farming. These type of data are sorely needed to understand the implications of the expansion of small-scale poultry farming in developing countries currently promoted as an economic development strategy.

This study utilized unique interdisciplinary methods to provide insights into the ecology of AR at the interface of humans and animals. First, unlike most environmental studies that summarize AR using clinical antibiogram methods, we used modeling techniques to better classify isolates into resistant and susceptible bacterial populations. Our approach is not suitable for classifying clinical isolates as it disregards pharmacokinetic and pharmacodynamics propertie, but may be better-suited for ecological studies. Although our conclusions were not sensitive to the use of custom susceptibility criteria, we show that the use of clinical breakpoints may misclassify over 30% of isolates in some instances, potentially leading to problems in the interpretation of results.

Further, we show that the use of the full phenotypic resistance profile (i.e., the full distribution of zones of inhibition or minimum inhibitory concentrations) represents a cost-effective way to study the epidemiology of drug-resistant strains in the absence of molecular data (Krumperman 1983; Sayah and Kaneene 2005). Analyzing the distribution of zone diameters in addition to categorical interpretations allowed us to identify a signature phenotypic pattern unique to production birds that suggest that AR associated with poultry production likely originates outside of the studied communities. A broader

implication of these findings is that focusing on poultry hatcheries and sources along the distribution chain may be more important than local management practices in controlling the spread of AR associated with small-scale poultry farming.

Finally, our ethnographic and survey data allowed us to characterize the variability inherent to the small-scale poultry farming in this region. Many of the drivers of the "boom and bust" nature of the enterprise that we identified through ethnography, such as access to capital and seasonality in markets as barriers to reinvestment, are likely at work in other developing regions as well. The variability of intensity of poultry farming across space and time has implications for the spread of AR in this region, and likely plays a role in limiting the impact of farming to the immediate production environment. Future studies should consider incorporating information and methods from multiple fields into an eco-epidemiological framework.

CHAPTER II: IMPACT OF ANTIBIOTIC EXPOSURE AND STOCKING DENSITY ON THE GENOTYPE AND RESISTANCE PHENOTYPE OF *E. COLI* SHED BY BROILER CHICKENS – A FACTORIAL EXPERIMENT[2]

CHAPTER SUMMARY

Antibiotics are routinely used in animal husbandry for growth promotion and prophylaxis, promoting the development of antibiotic resistance (AR) and contributing to the emergence of multidrug-resistant organisms that threaten human health. A factorial experiment was conducted in a facility in Northwest Ecuador to describe the within- and between-host dynamics of AR among *Escherichia coli* isolated from broiler chickens raised under different antibiotic treatments and stocking densities. Three flocks (1a, 2a, 2b) of 72 newly hatched chickens were sequentially raised in single, 6-chicken and 34-chicken cages and administered tetracycline (flock 1a) or enrofloxacin (flocks 2a and 2b) via water and virginiamycin via food on days 9 and 24. Flocks 1a and 2a were raised on fresh litter, and flock 2b was raised on the litter used by 2a. We collected cloacal samples on days 1 (prior to caging), 19-21 and 38 and tested three isolates against a panel of 12 drugs. The presence of four resistance genes (tetA, tetB, int1, qnrb) was determined using dot-blot hybridization. Isolates were genotyped on a microarray platform with 28 markers. Flocks arrived at the facility colonized with genotypically and phenotypically different communities of *E. coli*. The addition of antibiotics did not affect the prevalence of resistant phenotypes and genes, and

[2] Appears as Braykov NP, Zhang L, Cevallos W, Burbano N, López N, Marrs C, Foxman B, Trueba G, Eisenberg J, Levy K. Impact Of Antibiotic Exposure And Other Management Practices On The Phenotypic And Genotypic Composition Of Drug-Resistant E. Coli In Broiler Chickens (Poster #8). The 11th Annual DSAC Student Research Symposium. January 2014. Atlanta, GA;

Currently in preparation for submission.

the genotypic profile of communities. Increasing stocking density did not increase the prevalence of resistant phenotypes and genes. However, the genotypic profiles of chickens raised in single cages diverged from those that shared cages with 6 or 34 birds. There was a turnover of genotypes and phenotypes between each of the three sampling time points, particularly from first to second. Resistance genes were strongly associated with some resistance phenotypes and played a role in shaping community dynamics. Broilers that arrive pre-colonized with strains carrying integrons are more likely to carry such strains prior to slaughter, as well as strains phenotypically-resistant to sulfonamides and aminoglycosides. We conclude that chickens come pre-colonized with resistant strains and acquire the profile of the environment that they are brought into. The effect of management practices on the development of resistance at temporal scales of one flock are limited. Future studies should control for initial strain make-up, assess resistance at more frequent time intervals following drug administration, look at multiple host generations over longer time frames, multiple species or metacommunities of gut bacteria..

INTRODUCTION

The overuse and misuse of antibiotics hastens the evolution of antimicrobial resistance in human pathogens (Goossens et al. 2005), commensals (Marshall, Ochieng, and Levy 2009) and the environment (Martinez 2009). Infections with antibiotic-resistant bacteria are a growing public health threat, contributing to over 23,000 deaths per year in the United States (US Centers for Disease Control and Prevention 2013).

While efforts are focused on improving prescribing practices in the clinical context (MacDougall and Polk 2005), insufficient attention is being paid to antibiotics used in the intensive rearing of food production animals (Hollis and Ahmed 2013), which constitutes over 80% of antibiotic volume sold in the US (US Food and Drug Administration 2012). Agricultural use exerts selection pressure that can have an important impact on the evolution of resistance in commensal bacteria in human and animal hosts (Smith et al. 2002; Marshall and Levy 2011b).

The use of antibiotics in farmed animals has been associated with higher frequency of colonization with resistant bacteria (da Costa et al. 2011; Bager et al. 1997; van den Bogaard et al. 2001), and livestock production has been implicated in the emergence of clinically-relevant resistant pathogens(Rodri et al. 1999; Price et al. 2012). Resistant bacteria can be transmitted to humans via foodborne transmission on retail meat (Vincent et al. 2010; Schroeder, White, and Meng 2004), occupational exposure in farm workers and surrounding communities (van den Bogaard et al. 2001; S. B. Levy, FitzGerald, and Macone 1976), interactions with wildlife (Radhouani et al. 2014) and environmental pathways (Wellington et al. 2013; Silbergeld, Graham, and Price 2008)

Commercial poultry accounts for the largest share of livestock production in the Unites States (United States. Dept. of Agriculture. Foreign Agricultural Service 2014) and is rapidly growing in developing countries (Chang 2003). Going back to the foundations of modern animal production the 1940s, the poultry industry was the first to adopt antibiotics for the non-therapeutic purpose of growth promotion (Jones and Ricke 2003), and is currently the 2nd largest consumer of agricultural antibiotics worldwide with global demand projected to increase by 67% by 2050 (Boeckel et al. 2015). Over 30 compounds have been approved as feed additives in the rearing of broilers and laying hens, including anticoccidal agents indicated exclusively for veterinary use, bust also tetracycline, penicillin, chloramphenicol, fluoroquinolones, which are still used in the treatment and prevention of human infections (Jones and Ricke 2003; Dibner and Richards 2005).

The rationale for adding sub-therapeutic doses of antibiotics to poultry feed is twofold: first, antibiotic increase feed-conversion ratios by altering gut microbiota and decreasing competition with the host for nutrients (Dibner and Richards 2005; Smirnov et al. 2005), and by affecting the physical development of the chicken's intestinal system (Miles et al. 2006). Second, they prevent disease and lower mortality that arises in the crowded conditions typical of intensive farming (Dibner and Richards 2005; da Costa et al. 2011). In 2012, the average stocking density of broiler flocks in the US was 0.84 ft^2 ($\sim 0.08 m^2$) per chicken placed (United States. Dept. of Agriculture. Animal and Plant Health Inspection Service 2013). High stocking density increases disease transmission by making contact more frequent, and by increasing susceptibility to infection as a result of stress (Dibner and Richards 2005).

Existing research on poultry has separately investigated the links between resistance and subtherapeutic antibiotics or stocking density (Guardia et al. 2011; Funk et al. 2007),

and tends to focus on foodborne pathogens such as *Campylobacter* spp (van Boven et al. 2003; Avrain et al. 2003; Carrique-Mas et al. 2014) or *Salmonella* spp. (Carrique-Mas et al. 2014; Campioni, Zoldan, and Falcão 2014; Schwaiger, Schmied, and Bauer 2008). In the current study we focus on *Escherichia coli* as representative of commensal microbiota, which acts as an important reservoir in the promulgation of resistance genes (da Costa et al. 2008; Pleydell et al. 2007).

We report on results from a factorial experiment in Northwest Ecuador that was designed to address how modifiable management practices (antibiotics, cage density, litter change) affect within- and between-host population dynamics of drug-resistant *E. coli* in industrially farmed broilers. *A-priori*, we expect that:

1. Across flocks, chickens start with same level of low or no phenotypic resistance, and similar genotypic makeup as they all come from the same source.
2. Broilers whose water was supplemented with antibiotics active against *E. coli* will have higher prevalence of resistant phenotypes and resistance genes, as antibiotics in the treatment group exert selection pressure .
3. Treatment will affect the genotypic make-up of commensal flora and clear the majority-susceptible strains, decreasing the diversity of *E. coli* genotypes and making communities dissimilar across treatment groups.
4. Prevalence of resistant phenotypes and resistance genes will be higher in cages with more animals, as the high stocking density increases the opportunity for the sharing of resistance genes.
5. Genotypic diversity will be associated with high stocking densities at later days, as animals living closer together have more opportunities to exchange strains of *E. coli.*

6. Genotypic dissimilarity between stocking density treatments will be significant and will increase at later time points, as communities are formed in separately caged animals and under different ecological conditions.

METHODS

Study design

Three flocks of 72 broiler chickens were consecutively raised between February and October 2010 at a poultry farming facility in the Esmeraldas region of Northern Ecuador. The facility, feed and bird stock were supplied by the Procesadora Nacional de Alimentos C.A. (PRONACA), an integrated poultry manufacturer that is among the largest in the country.

To test the hypothesis that non-therapeutic antibiotic use selects for resistant enteric bacteria, we designed each trial as a balanced factorial experiment where newly hatched chicks were randomized to four treatment groups of 18 birds each: control, antibiotic treatment in water, antibiotic treatment in feed, and antibiotic treatment in both water and feed.

The first flock (trail 1a) received tetracycline treatment in water and was raised on fresh litter, the second (trial 2a) received enrofloxacin in water and was raised in fresh litter, and the third (trial 2b) received enrofloxacin in water and was raised on the litter used by the 2a trial. Date from a fourth trial with tetracycline and used litter was never collected as the entire flock was infected with *Mycobacterium* and had to be sacrificed prematurely. The antibiotic added to the feed was virginiamycin, which has no activity against *E. coli.* Antibiotics were administered in routine doses and intervals recommended by PRONACA: on days 9 and 10 and 23 and 24 of the growth cycles, 5g/ton of virginiamycin were added to the feed mixture, and solutions of 5mg/kg of tetracycline and 7.5 mg/kg of enrofloxacin were dissolved in the daily water dose (5ml dissolved in 10l of drinking water

on days 9-10, 20ml in 20l on days 23-24). All birds, including controls, received monensin (90ppm concentration at 110g/ton) in their feed as anticoccidial prophylaxis.

To test the hypothesis that higher stock density increases the potential for chicken-to-chicken transmission of resistant strains/sharing of genotypes, in each treatment group we raised 6 birds in single cages, 6 together in a single enclosure of the same size, and 6 in a group of 34 raised in the same enclosure. Cage sizes were 55X55cm (~0.3m^2 per chicken) for single birds, 100x100cm for 6-chicken enclosures (1m^2 or ~0.16m^2 per chicken), 187x180cm (3.3m^2 or ~0.1m^2 per chicken) for 34-chicken enclosures.

Sample processing

Cloacal samples were obtained on days 1 (prior to placement in cages), 19-21 and 38 (pre-slaughter) and were plated for isolation on Chromocult Agar (Merck, Darmstadt, Germany). Three distinct colonies were selected at random and transferred to MacKonkey Lactose Agar, and were then transferred back to Chromocult tri-petri dishes to ensure pure cultures. Samples were also plated on agar enriched with tetracycline and enrofloxacin to select for isolates resistant to each antibiotic. One colony per sample per enriched media was randomly selected for further testing.

Antibiotic susceptibility testing

Susceptibilities to beta-lactams (BL, incl. ampicillin, amoxiclav, cefotaxime, ceftazidime, caphalotin), aminoglycosides (AG, incl. streptomycin and gentamicin), fluoroquinolones (FQ, incl. ciprofloxacin and enrofloxacin), sulfonamides (S), chloramphenicol (C) and tetracycline (TE) were measured using standard Kirby-Bauer disk diffusion methods (Bauer et al. 1966) with commercially prepared discs (Becton Dickinson, Franklin Lakes, NJ).

Genotyping

Isolates were fully genotyped based on presence/absence of 28 genes using a high-throughput dot-blot hybridization on the Library-on-a-Slide (LOS) array platform developed previously in our laboratory with internal gene probes prepared with primers listed in (Zhang et al. 2004). Hybridization conditions and the analysis of the probing results have been described in detail elsewhere (Kong et al. 2006; Zhang et al. 2012). In addition to the randomly chosen 28 genes, the arrays assessed the presence of class-1 integrons (int1), plasmid-mediated quinolone resistance (qnrb), and two tetracycline genes (tetA and tetB).

Statistical analysis

Our main outcomes of interest were 1) the proportion of resistant isolates to each antibiotic class based on the categorical interpretation (susceptible or non-susceptible) of the zone of inhibition; 2) the prevalence of resistance genes (int1, qnrb, and tetA or B combined as a single variable); 3) the Bray-Curtis dissimilarity and Shannon diversity indices of genotypes collapsed at the chicken and sample levels.

Phenotypic resistance and gene prevalence

To classify isolates as phenotypically resistant or susceptible, we derived custom breakpoints to each of the 12 drugs by fitting two-component mixture models to zone diameter distributions of production bird isolates to index isolates into susceptible and resistant populations. An isolate was considered resistant to an antibiotic class if it was classified as non-susceptible to one or more of the test drugs belonging to that class.

To fit a mixture model, we compared the fits of 3 expectation maximum algorithms from R's mixtools package (Benaglia et al. 2009): a parametric, semi-parametric and non-

parametric specification that accounted for repeated measurement data to account for multiple isolates cultured from each sample. Among those, the best-fit model was selected based on the log-likelihood statistic. Custom breakpoints were set where the density estimates for the fitted distributions intersect. These updated breakpoints were rounded to the nearest whole number and used to categorize all isolates in our data as susceptible and resistant. Custom susceptibility breakpoints that may more accurately reflect the local population of bacterial strains compared to externally defined breakpoints based on wild-type cutoff values (Wiggins 1996). These custom breakpoints might have limited clinical utility, but are more consistent with the objective of our study to describe the local ecological dynamics of *E. coli* with regard to antibiotic susceptibility.

We compared the percentages of non-susceptible isolates and of gene prevalence, across categories of interest (day of sampling, cage density, trial, sample type etc.). To account for the hierarchical, unbalanced nature of the data when comparing across categories, we used generalized linear mixed models (GLMM) with a binomial link function to model the outcome of AR fitted by maximizing a Restricted Maximum Likelihood Criterion at convergence in R's lme4 package (Bates, Maechler, and Bolker 2013). Univariate, bivariate and bivariate models with interactions were assessed. The best-fit model was chosen to minimize the Akaike Information Criterion. When modeling phenotypic resistance, a separate regression was considered for each drug class as resistance mechanisms to the tested drug classes are independent. In all specifications, we used nested random effects with varying nested intercepts by sample and bird to capture unobserved heterogeneity between the multiple levels and account for repeated sampling.

Genotypic profile

The 28 gene probes included in the microarray protocol were randomly chosen, but could still exhibit a substantial degree of correlation. To avoid biased comparisons of diversity and similarity, we clustered the markers based on their expression (as a binary value) across the entire set of isolates. We used the BHC Hierarchical Bayesian clustering package in R, designed to automatically determine the optimal number of clusters in microarray data (Savage et al. 2009). The method performs bottom-up (agglomerative) hierarchical clustering, using a Dirichlet Process (infinite mixture) to model uncertainty in the data, and Bayesian model selection to decide at each step which clusters to merge clusters (Savage et al. 2009).

Alpha Diversity within groups

To measure the genotypic diversity of communities with a measure of uncertainty, we computed a bootstrapped Shannon-Weiver index (H) for different subsets of the data (Jost 2006). First, we constructed a species-site matrix for the presence/absence of clustered genotype markers across samples. We aggregated these data from the isolate to the sample and chicken levels, summing the abundance of clusters in each sample. This matrix was passed to the H.value function in R's vegetarian package (Charney 2015), which uses a bootstrapping procedure with n=500 iterations to compute mean and standard error estimates for H (Chao 2004).

To describe and visualize the richness and evenness of genotypic profiles of isolates, and map their correspondence to the gene and phenotype profiles, we used a parallel axis produced by a modified version of R's function ggparallel (Hofmann and Vendettuoli 2015).

Beta-diversity and dissimilarity across factors

To reduce dimensionality in the genotype data and visualize the genotypic similarity between samples across different categories, we performed an unconstrained ordination using Nonmetric Multidimensional Scaling (NMDS). We aggregated the species-site matrix from the isolate to the sample and chicken levels, summing the abundance of clusters in each sample, and computed a matrix of Bray-Curtis pairwise dissimilarities that was passed as an argument to the metaMDS function in R's vegan package (Oksanen et al. 2012).

To partition the genotypic variance among multiple factors in the experiment, we used an analysis of dissimilarity model (Adonis), a multivariable permutation test for fitting linear models to distance matrices (Oksanen et al. 2012). The treatment group, stocking density, day of sampling and gene profile were added to the model in that order. The software performs n=1,000 permutations stratified by trial and produces a table with pseudo-*F* statistics and a corresponding *P*- and *R*-squared values for each covariate.

RESULTS

Sample characteristics

A total of 1683 *E. coli* isolates were cultured from 575 samples obtained from 215 broilers (Table 1) raised in the periods February 2 – March 11, 2010 (trial 1a), June 24 – August 3, 2010 and 10 September– 16 October, 2010 (trial 2b). The sample yield is lower than the theoretical maximum as *E. coli* was not successfully isolated from all chickens at all sampling points. In addition, X samples for which there were no genotypic data available were dropped from the analysis.

Table 4: Number of chickens, samples and isolates in each flock

Trial	Chickens	Samples	Isolates
1a tetracycline/new litter	72	191	555
2a enrofloxacin/new litter	71	187	555
2b enrofloxacin/used litter	72	197	573

Prevalence of resistance over time and treatment group, by flock

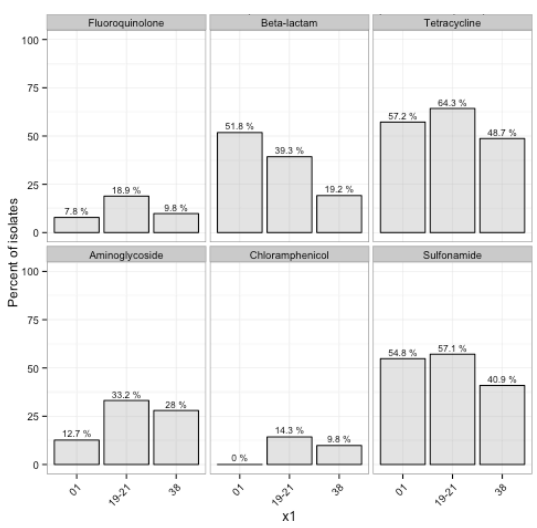
The proportion of resistant isolates from samples taken over the broilers' life followed a different trajectory across trials (Figure 1). In trial 1a, comparing pre-slaughter (day 38) to pre-caging (day 1), it decreased for beta-lactams ($P<0.01$), sulfonamides ($P<0.05$), showed no change for tetracycline ($P=0.48$) and fluoroquinolones. The GLMM univariate model did not converge for chloramphenicol due to rare occurrence of the outcome. In trial 2a resistance increased significantly for all drug classes, and started with very low levels, particularly for flouroquinolones and beta-lactams (0.7% and 8.7%, respectively). In contrast, in trial 2b levels of resistance were very high (>50% for fluoroquinolones, sulfonamides, beta-lactams and tetracycline) before the chicks were even placed in cages, and decreased for sulfonamides, fluoroquinolones and beta-lactams ($P<0.001$), increased for aminoglycosides and chloramphenicol ($P<0.001$) and did not change for tetracycline ($P=0.41$).

Contrary to our hypothesis, the proportion of resistant isolates across treatment groups relative to antibiotic-free controls differed on day 1, and overall differences and diminished by day 38 (Figure 7). In bivariate models with sample time and treatment

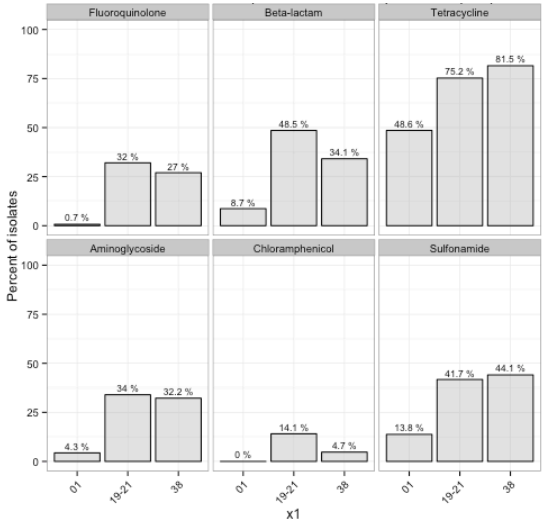
group (forest plots shown in Supplementary figure 8), time was significant in all trials in all models (P<0.001), and treatment factors were generally not, with the exception of lower resistance among Water treatment among sulfonamides, fluoroquinolones and chloramphenicol in trial 2a, and lower resistance in Water, Food and Both among fluoroquinolones in trial 2b. Testing for treatment variable and time interactions did not improve model fit, with the exception of a significant interaction between time and treatment group for sulfonamides in trial 1a, where chickens in the Both and Food groups had higher resistance relative to controls on days 19 and 21, but those just in water did not (*P*=0.32), and a similar effect for sulfonamides in trial 2b.

Figure 6: proportion of resistant isolates over time, by trial

A) Trial 1a (tetracycline/fresh litter)



B) Trial 2a (enrofloxacin/fresh litter)



C) Trial 2b (enrofloxacin/used litter)

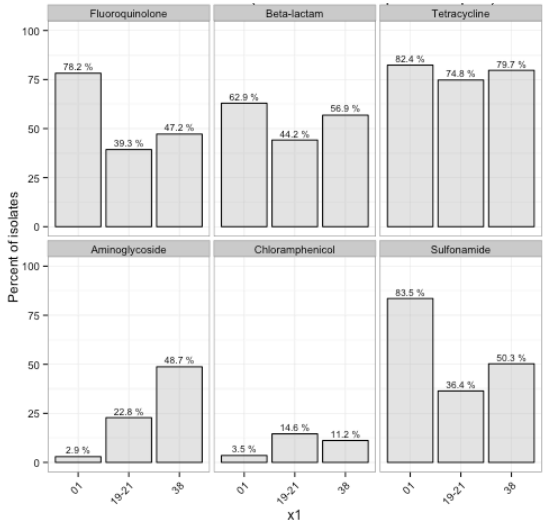
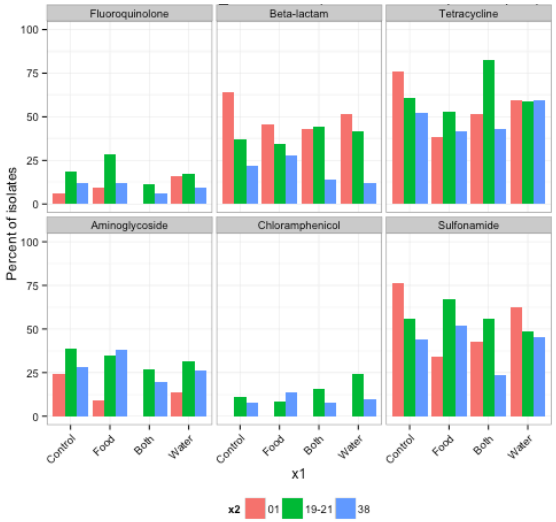
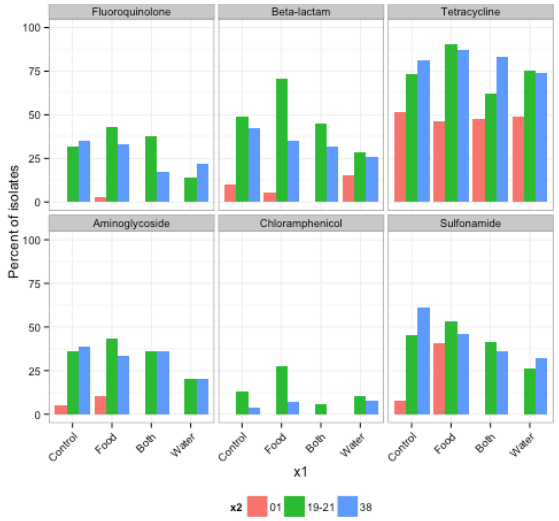


Figure 7: proportion of resistant isolates over time and across treatment groups, by trial

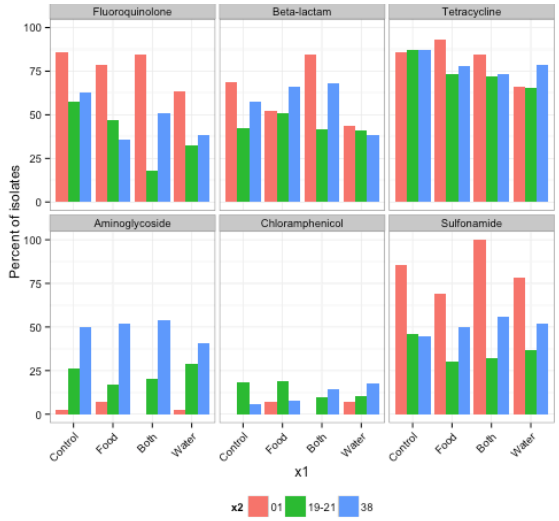
A) Trial 1a (tetracycline/fresh litter)



B) Trial 2a (enrofloxacin/fresh litter)



C) Trial 2b (enrofloxacin/used litter)

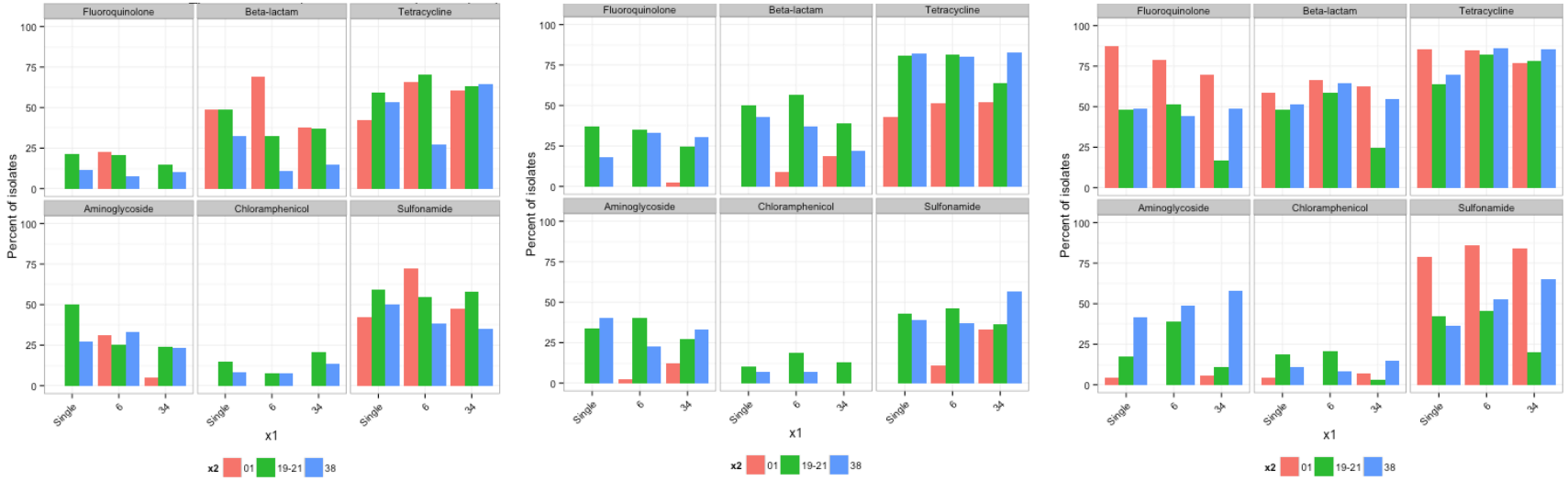


Prevalence of resistance over time and stocking density, by flock

There were no differences in the proportion of resistant isolates across stocking densities over time (Figure 8). In bivariate models with sample time and treatment group (), fixed effects for time showed the same significance and direction as in univariate models. Cage densities were generally not associated with resistance. In trial 2b the 34- and 6-chicken cages started with a higher resistance to sulfonamides and beta-lactams on day 1. However, in GLMM models, thirty-four chicken cages had a higher resistance only to sulfonamides. Testing for interactions did not improve model fit in any of the trials, with the exception of sulfonamides in trial 2b where on day 38 there were significantly more resistant isolates ($P<0.05$) in 34-chicken cages than in singles.

Figure 8: proportion of resistant isolates over time and across stocking density groups, by trial

A) Trial 1a (tetracycline/fresh litter) B) Trial 2a (enrofloxacin/fresh litter) C) Trial 2b (enrofloxacin/used litter)



Association between phenotypic resistance and gene carriage

Associations between phenotypic resistance and gene prevalence for pooled data are shown in Figure 9. Carriage of int1 was associated with resistance to all drugs (*P*<0.001), particularly sulfonamides and fluoroquinolones. Predictably, tetA or tetB carriage was highly predictive of tetracycline resistance and was also associated with elevated levels of resistance to all classes (*P*<0.001). Surprisingly, qnrB carriage was either not significantly related, or was protective of resistance to fluoroquinolones, sulfonamides, beta-lactams and aminoglycosides (*P*<0.001).

Association between resistance genes

In pooled data from day 1 of all trials, the presence of int1 was very strongly associated with carriage of tet A or tetB (univariate OR 3092.4, 95% CI 493–15,343 with random effect for bird), negatively collinear with qnrB; tetAB was negatively associated with qnrB presence (univariate OR 0.041, 0.01– 0.1). When all time points were considered, those associations became weaker (univariate ORs of 3.43[2.1–4.34] for int1 and qnrB, 0.18 [0.06–0.4] and non-significant association for tetAB and qnrB).

Prevalence of resistance genes over time and across trials

As expected from the gene-phenotype associations outlined above, the prevalence of resistance genes over time and across treatment groups (A) followed the same patterns as the relevant phenotypes. In trial 1a ~75% of the control group isolates had int1 on day 1, compared to just under 50% in the other groups, which manifested itself in high phenotypic resistance to sulfonamides, fluoroquinolones and aminoglycosides in that group (Figure 7A). Similarly, in trial 2a, there were no int1 isolates on day 1, and an unusually high prevalence of qnrb (>80%, B), which manifested itself in very low resistance to fluoroquinolones, beta-lactams and sulfonamides (Figure 7B). In contrast, in trial 2b there

was very high carriage of int1 and tetAB on day1 (C), particularly in the Control and Both groups (~75%), which mapped to high levels (>75%) of sulfonamide, beta-lactam, fluoroquinolone and tetracycline resistance (Figure 7C).

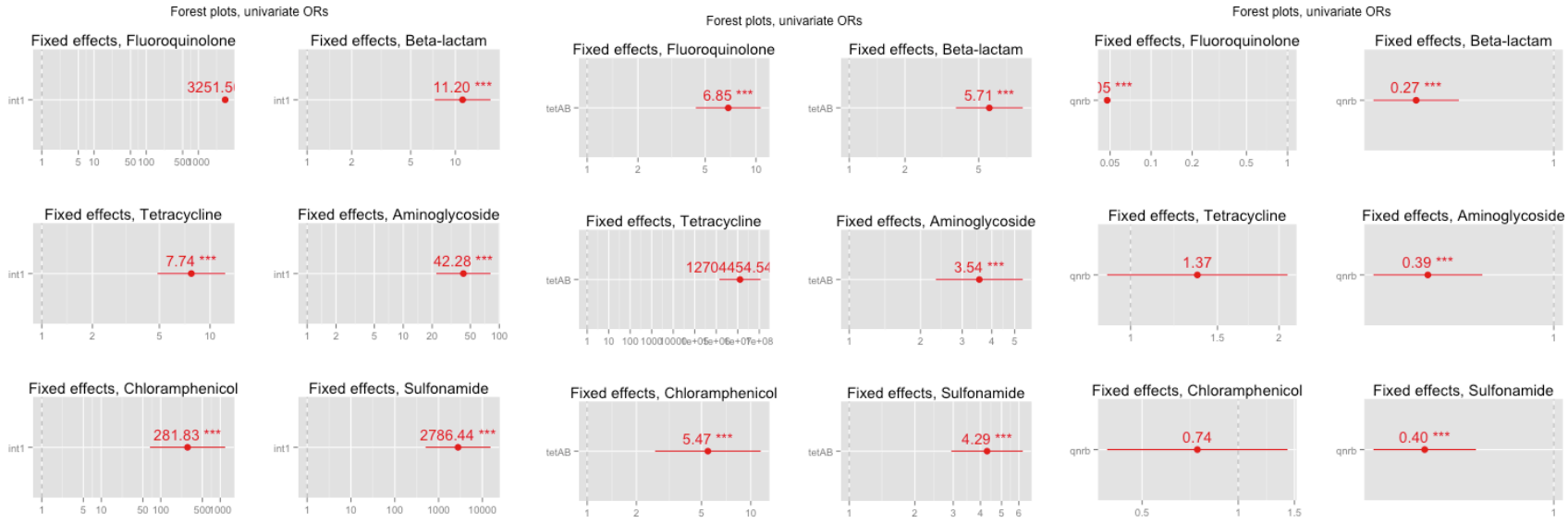
Effects of pre-colonization with integrase 1 on pre-slaughter resistance

To test a secondary hypothesis that pre-colonization with int1 is linked to high resistance when they are slaughtered, we used univariate GLMM models on the pooled data to assess the univariate odds of isolating resistant *E. coli* on day 38 if a chicken tested positive for int1 on day 1. While all estimated effects were positive, the effects were significant for aminoglycosides (OR 6.39, 2.01–19.97) and sulfonamides (OR 2.01, 1.07–3.87). In addition, int1 on day 1 was predictive of int1 on day 38 (OR 2.31 1.21–4.8).

Figure 9: forest plots of univariate associations of phenotypic resistance and carriage of resistance genes

Forest plot showing Odds Ratios and 95% Confidence Intervals from Generalized Linear Mixed Effect Models fitted by maximizing a Restricted Maximum Likelihood Criterion with nested random effects for sample and chicken. Colors reflect the direction of the effect. Type 3 like *p*-values calculated using an approximation for degrees-of-freedom. Estimates with *P*-values <0.05 are in bold, and are annotated with stars corresponding to *P*<0.05(*), *P*<0.01(**), *P*<0.001 (***). Intercept estimates not shown.

A) int1 B) tetAB C) qnrb



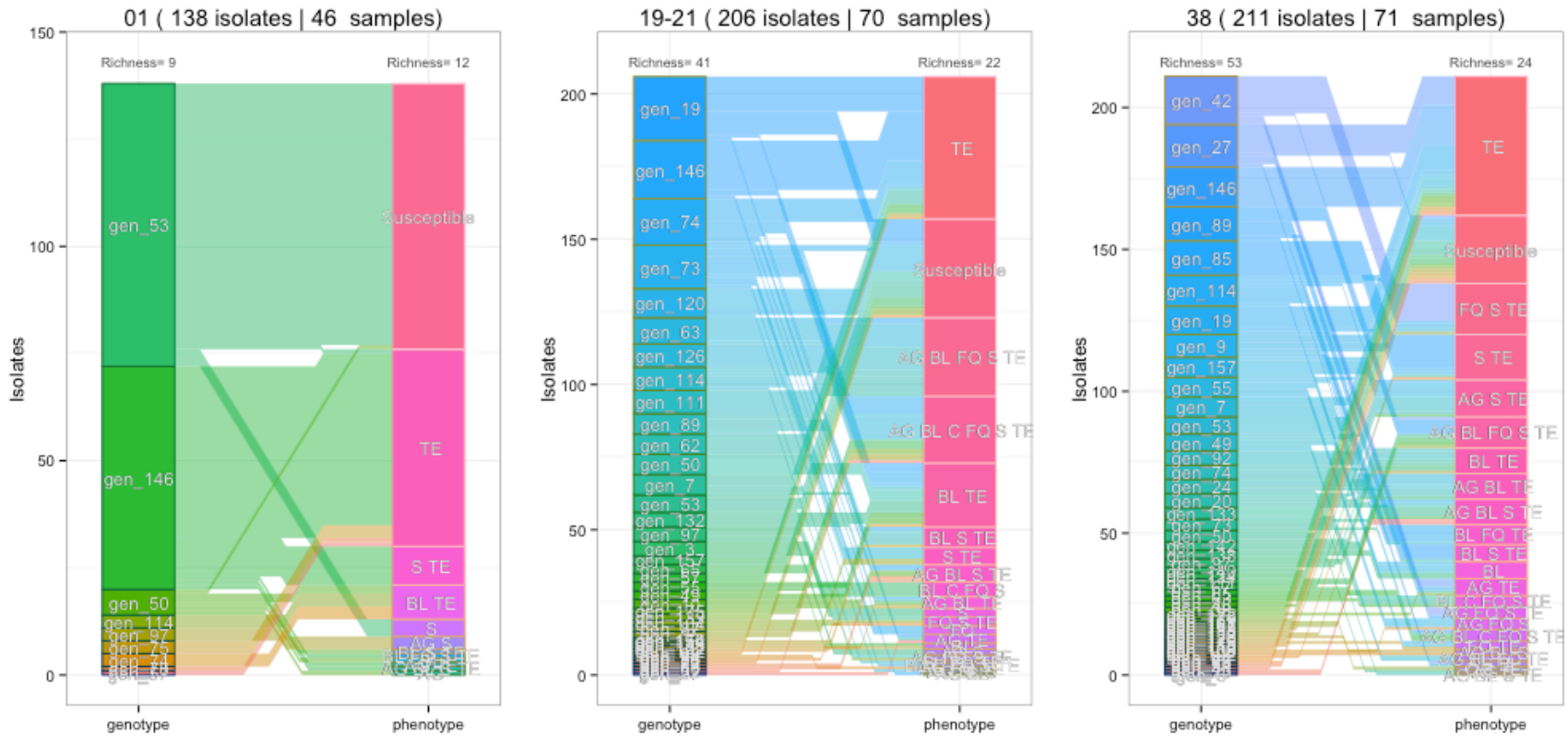
Phenotypic and genotypic dynamics over time, by trial

A parallel axis plot was used to visualize the dynamics of phenotype and genotype abundances over time and across trials, as well as the correspondence between each phenotype and genotype (Figure 10). Across trials, genotypes on day 1 had and uneven abundance of 9–13 unique genotypes, with clear correspondence to 12–14 phenotypes. Dominant genotypes were unique to trials. In subsequent sampling points, the richness of both phenotypes and genotypes became more even and increased considerably with 41-42 genotypes corresponding to 22-26 phenotypes. Unique genotypes no longer corresponded to singular, unique phenotypes.

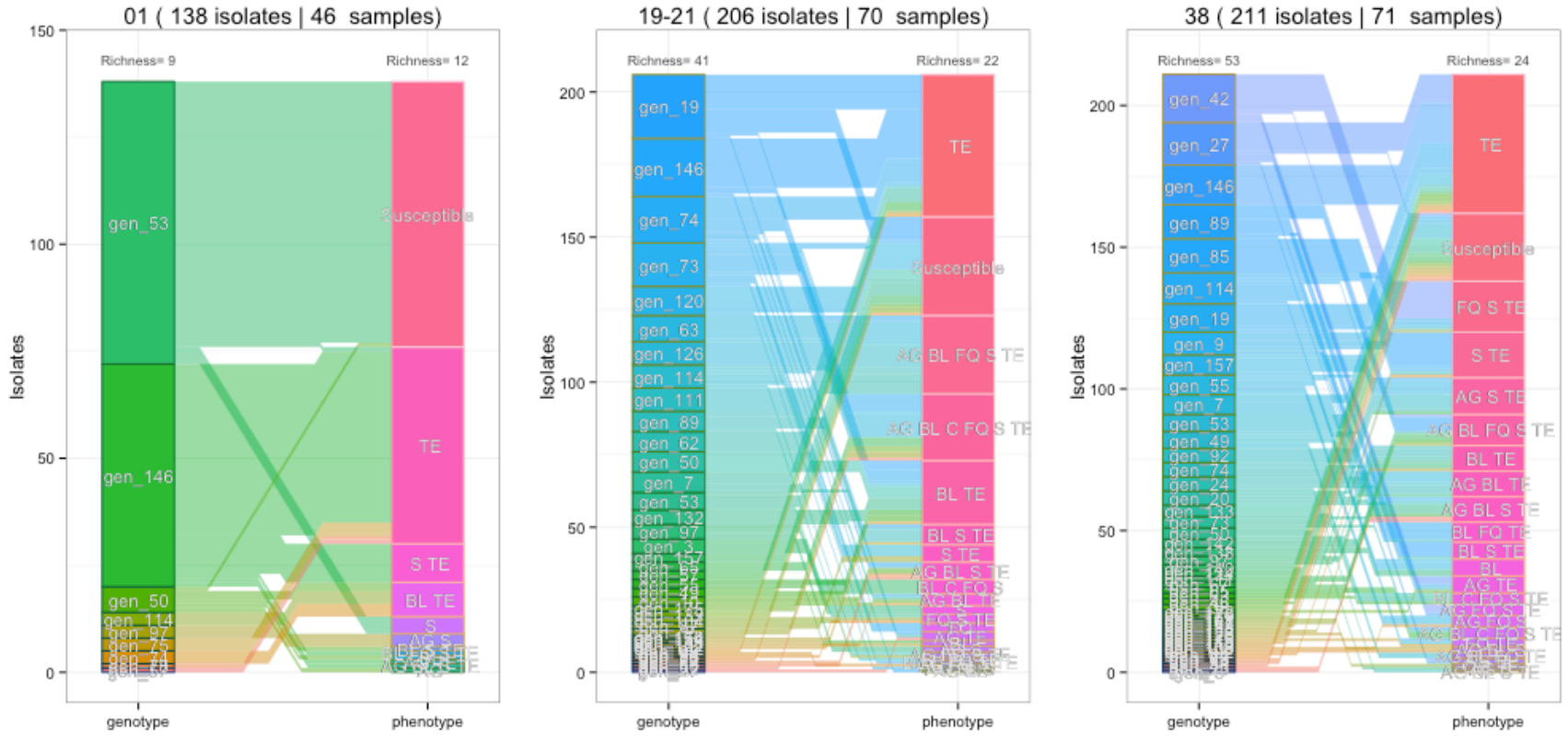
Figure 10: proportion of resistant isolates over time and across stocking density groups, by trial

The parallel axis plot shows the relationships between the abundance of genotype and phenotype over day of sampling, where the axes between the columns link corresponding observations in each category. Each block is colored separately with colors chosen from spectral palette. Each panel is a sampling day.

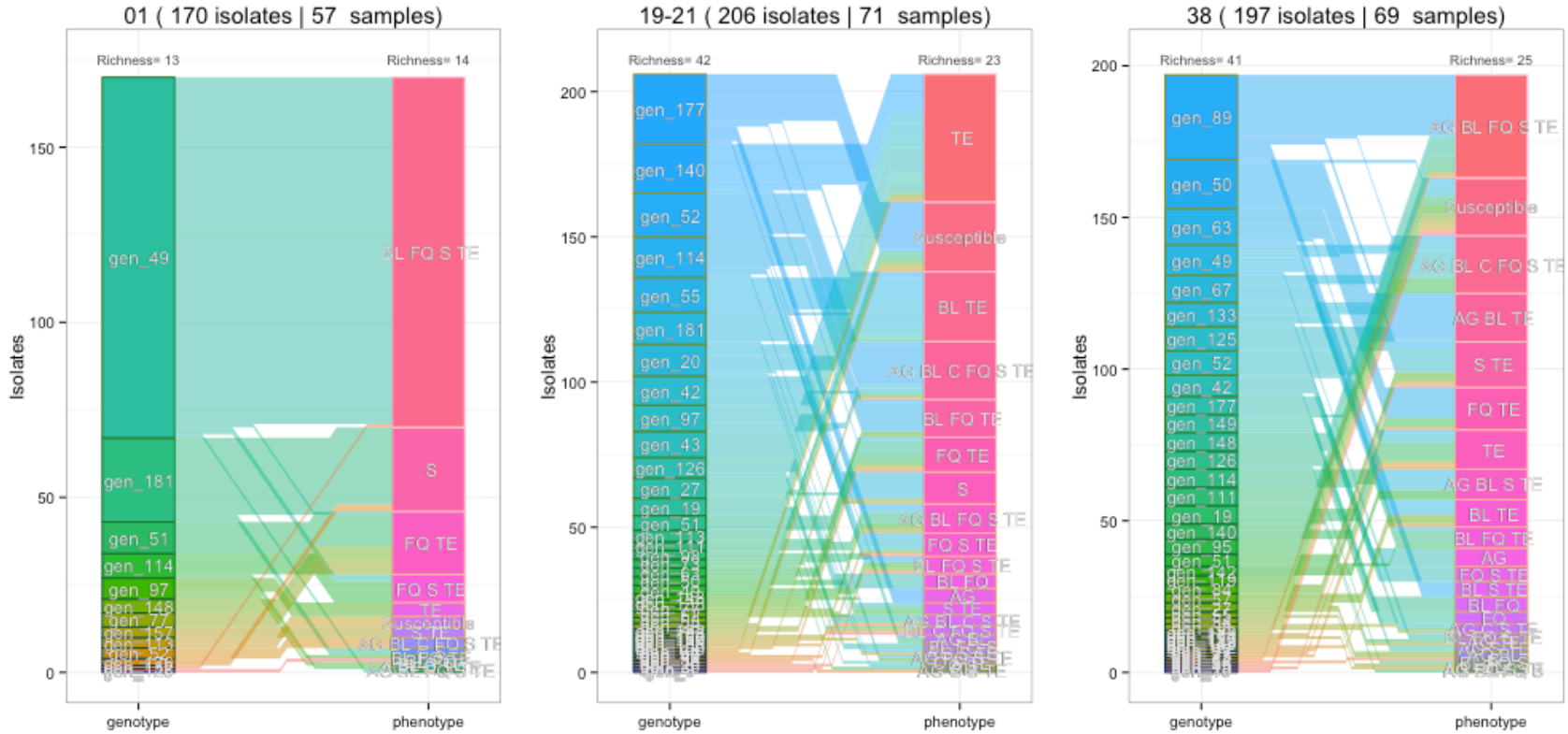
A) Trial 1a (tetracycline/fresh litter)



B) Trial 2a (enrofloxacin/fresh litter)



C) Trial 2b (enrofloxacin/used litter)



Diversity dynamics by treatment and cage density

Contrary to expectations, the diversity of genotypes measured as average Shannon index (H) within treatment groups (**Figure 11**) for the pooled data from all experiments increased from day 1 by ~40% and remained at similar levels on the subsequent sampling points. While there were differences between treatment groups on day 1, those did not persist at later time points. Similarly, the diversity of genotypes within cage density groups (Figure 12) for the pooled data did not differ between the cages at later time points.

Figure 11: Dynamics of genotypic diversity within treatment groups

Error bars are 95% bootstrap confidence intervals calculated according to (Chao 2004).

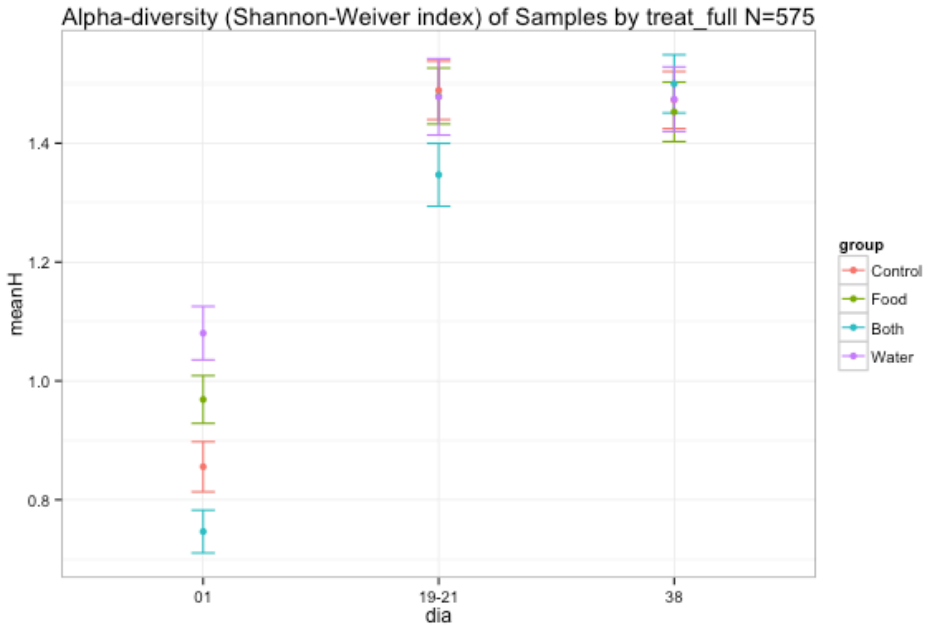
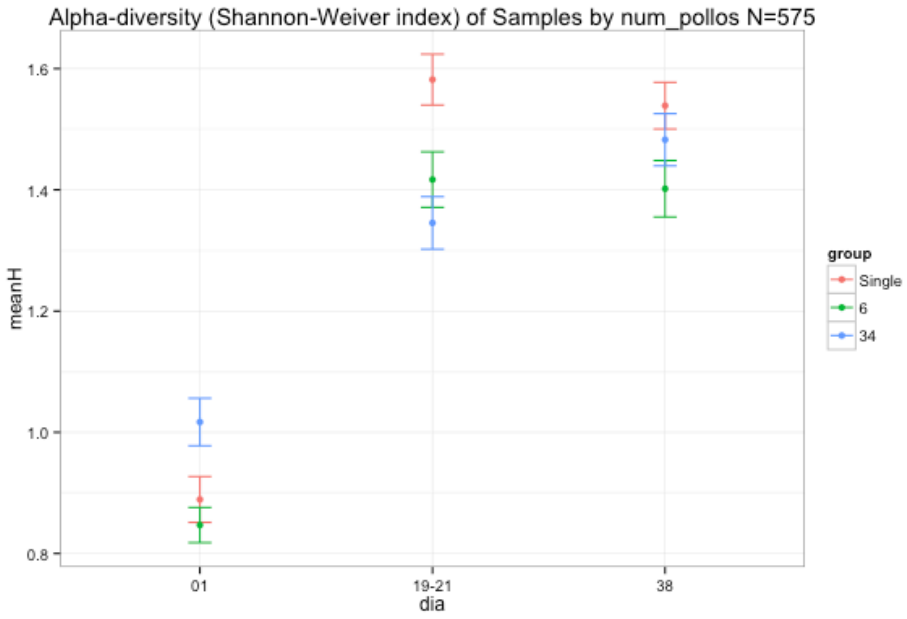


Figure 12: Dynamics of genotypic diversity within stocking density groups

Error bars are 95% bootstrap confidence intervals calculated according to Chao (2004)



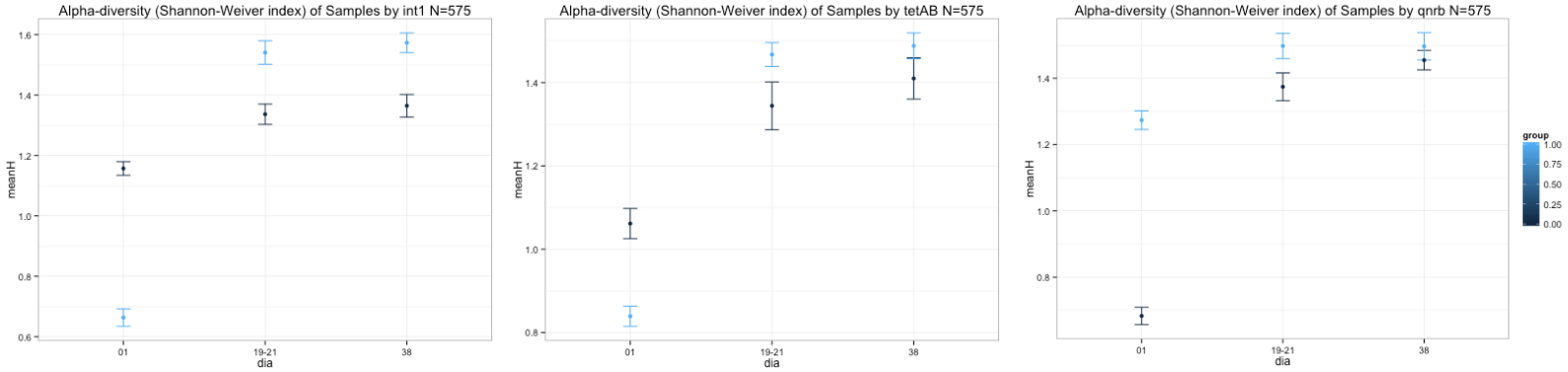
Diversity dynamics by presence of resistance genes

The Shannon diversity of pooled data from all samples followed different temporal patterns based on the presence of resistance genes (Figure 13). On day 1, samples that were positive for int1 were over three times less diverse on average as those without the gene. The difference was of lesser magnitude for tetA or tetB and of opposite direction for qnrB. The proportion of isolates carrying a gene in each timepoint was not drastically skewed: 78/279, 114/294 and 178/286 samples were positive on days 1, 19-21 and 38 for int1, 104/279, 176/294 and 169/286 for tetA/tetB, and 64/279, 125/294 and 97/286 for qnrb.

Figure 13: Dynamics of genotypic diversity, by presence of resistance genes

Error bars are 95% bootstrap confidence intervals calculated according to Chao (2004)

A) int1 B) tetAB C) qnrB



Genotypic dissimilariy between experimental trials on different days

The genotypes were dissimilar across trials (P<0.001 for all restricted and unrestricted comparisons) , and although the magnitude of the dissimilarity was largest when the sample was restricted to day1 (R-sq=0.26), it was still significant in the subsequent sampling time pointe (0.04 and 0.052),

Genotypic dissimilariy between sampling days

Of the variation in the genotype that is explained by the sample day variable, the vast majority is from day 1. There were significant differences (P<0.001, R-squared 0.096) between the genotypes in each of the 3 sampling days in an Adonis model with N=1000 permutations stratified by trial. When the sample was restricted just to days 19 -21 and 38, the significance of the difference decreased (P =0.035), as did the proportion of explained variation (R-sq=0.00576). The test was stratified by trial because the results in the preceding section showed trials have different genotypes, and we can assume each trial was independently conducted.

Genotypic dissimilariy between treatment groups on different days

Treatment group did not explain a significant proportion of the variation (P=0.35, R-squared 0.004) in the pooled sample stratified by time and trial. Results were similar when the sample was restricted to each time point. The test was stratified by trial and time point because the results in the preceding sections showed these variables explain a significant share of the variation.

Genotypic dissimilariy between stocking density groups on different days

The stocking density explained a small, but significant proportion of the variation in genotype (R-sq = 0.0124, *P* <0.001), which increased over time. In restricted comparisons, the difference between the single and 34 and single and 6-chicken cages was significant (*P*<0.01). However, that between 6 and 34-chicken cages was not (*P*=0.08). In comparisons restricted to particular time points, there were no significant differences between stocking density groups on day 1 (*P* =0.19), but the difference was significant in the sample when day 1 was excluded (R-sq = 0.17, *P* <0.001).

Genotypic dissimilarity in a multivariable model

In the Adonis model adjusted for all relevant analysis variables (**Table 5**), treatment group (treat_full) did not explain a significant fraction of the variation genotypes. All other covariates did (P<0.001). The time of sampling (dia) explained approximately half of the explained variation (R-sq = 0.096), and the presence of resistance genes and stocking density group (num_pollos) accounted for roughly similar proportions of the variance.

Table 5: Multivariable analysis of dissimilarity (Adonis) model

Variables added in specified order, with N=1000 permutations stratified by trial

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)	
treat_full	3	0.490	0.1632	1.115	0.00491	0.322	
num_pollos	2	1.238	0.6189	4.228	0.01241	0.002	**
dia	2	9.678	4.8392	33.054	0.09705	0.001	***
int1	1	1.066	1.0661	7.282	0.01069	0.001	***
tetAB	1	2.397	2.3973	16.375	0.02404	0.001	***
qnrb	1	2.282	2.2820	15.588	0.02288	0.001	***
Residuals	564	82.571	0.1464		0.82801		
Total	574	99.722			1.00000		

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

DISCUSSION

In this set of factorial experiments, we investigated the effect of modifiable management practices – antibiotic administration and stocking density – on the dynamics of antimicrobial resistance and the genotypic makeup of *E. coli* communities in commercial broilers. We found that: 1) the three flocks arrived at the facility colonized with genotypically and phenotypically different communities of *E. coli*; 2) the addition of antibiotics did not affect the prevalence of resistant phenotypes and genes, or the genotypic profile of communities; 3) increasing stocking density did not increase the prevalence of resistant phenotypes and genes. However, the genotypic profiles of chickens raised in single cages diverged from those that shared cages with 6 or 34 birds; 4) there was a turnover of genotypes and phenotypes between each of the three sampling time points, particularly from first to second; 5) resistance genes were strongly associated with some resistance phenotypes and played a role in shaping community dynamics. Broilers that arrive pre-colonized with strains carrying integrons are more likely to carry such strains prior to slaughter, and be resistant to sulfonamides and aminoglycosides.

Contrary to our expectations, each of the three flocks came into the facility with different resistance phenotype and genotype makeup. The difference is particularly pronounced for experiments 2a and 2b, where the former flock had a complete absence of int1 and correspondingly minimal levels of fluoroquinolone and beta-lactam resistance, and the latter had very high levels. As sampling was conducted prior to the placement of chicks in their cages, this finding confounds flock-level comparisons and limits our ability to test for the effects of raising chickens on fresh versus used litter. The finding is also consistent with our observational study in 17 village communities in the same region, where broilers under 2 weeks of age and sourced from a particular veterinary store had very high levels of

resistance to broad-spectrum antibiotics. Difficulties in reproducing results of similar experimental designs with multiple flocks are also reported in the literature (Piddock et al. 2008), as is the role of hatcheries as sources of resistant bacteria(Musgrove et al. 2006). To avoid confounding from comparisons between experimental groups, future studies of flocks in open systems like ours could clear and recolonize the chicken's gastrointestinal tract prior to start of the experiment by giving high doses of antimicrobials and transplanting lyophilized intestinal microflora of specific pathogen-free chickens (van Boven et al. 2003).

More importantly, we found that prior to their slaughter, batches of chicks that had come from hatcheries carrying high levels of resistance genes were over twice as likely to continue to carry such genes and exhibit resistance to multiple antibiotic classes. This suggests that poultry from these facilities contributes to a well-studied reservoir of resistance in retail meat (Schroeder, White, and Meng 2004). Mobile genetic elements that confer resistance or aid in its transfer, such as integrases, have been found to pass from retail meat into the community (Leverstein-van Hall et al. 2011) and from the community to the hospital (Leverstein-van Hall et al. 2002). In addition, farm workers, their families and surrounding communities might be at higher risk of colonization with resistant strains if working with animals carrying resistant bacteria (Neyra et al. 2014; Rinsky et al. 2013).

We did not observe an expected increase in the prevalence of resistance in treatment groups, particularly those that were given antibiotics with activity against *E. coli* (enrofloxacin and tetracycline) in water, the route thought to exert the greatest selection pressure. Although most studies conclude that antibiotics select for more resistance (da Costa et al. 2009; Bonnet et al. 2009b), our finding is also consistent with published work specific to poultry and *E coli*: Diarra et al. (2007) worked with a flock of 900 chickens that were given several antibiotic growth promoters, including penicillin and salinomycin; the

authors report a decrease in the overall presence of resistance, and a high frequency of multi-drug resistance independent of the use of antibiotics. Van Boven et al. (2003) studied the within-host dynamics of *E. coli* and *Campylobacter jejuni* in a flock of 32 birds that was raised in isolators and sampled daily for 50 days. They found that sample counts for both species decreased after treatment with fluoroquinolones, but whereas there was an increase in the frequency of *C. jejuni*, that was not observed in *E. coli.* Lack of selection in other pathogens has also been reported in pigs treated with tetracycline (Funk et al. 2007; Oliveira et al. 2010)

There are several explanations for the lack of treatment effects we observed: first, as effects of antibiotics on gut microbiota can be highly transient and reversible(Robinson and Young 2010; Antonopoulos et al. 2009; Pleydell et al. 2007), it is possible that we missed a temporary amplification of the resistance population as we sampled chickens on days 19-21 and 38, which was more than 10 days following the addition of antimicrobials in water. Second, the antimicrobial dose we sued may not have been sufficient to produce an effect that can be captured by our sampling. Third, our system was not closed: although chickens were randomized into treatment and control groups, the air in the facility was shared and constantly circulated, were interacting in the non-single cages. It is possible that high resistance in antibiotic-treated chickens was transmitted to controls via chicken-to-chicken contact in 6 and 34-chicken cages. However, in this case we would expect a positive interaction between treatment group variables and cage density, which was not observed. Finally, observations on within-host dynamics of AMR are highly dependent on the microbiological method used. For instance, when an observational study from Denmark estimated the proportion of Vancomycin-resistant enterococcus in pigs by measuring sample-level bacterial population frequencies, as opposed to the frequency of cultured resistant isolates, the authors reached a new conclusion that VRE had persisted in Danish

pigs 5 years after the withdrawal of antibiotics from the agricultural system(Garcia-Migura et al. 2007).

Effects of selection could be ascertained more clearly if the microbiota is sampled mode widely, more frequently, or over a longer period of time. Future studies should look at multiple species, or preferably employ metagenomic approaches (Stanley, Hughes, and Moore 2014; Van Der Wielen et al. 2002; Sergeant et al. 2014; Danzeisen et al. 2011). If culture-based methods are used, bacterial counts should be performed to estimate the population size and estimate the frequency of resistant colonies at the level of each sample (Pleydell et al. 2007; van Boven et al. 2003). The high levels of resistance generally associated with intensive agriculture likely accumulate over longer periods of time than a single production cycle. Research looking at horizontally transferred microbial communities over multiple generations of animals can assess dynamics at these scales.

We also found limited effects of varying stocking density on the prevalence of resistance. The dilution of the hypothesized effect could also be due to the openness of the system, and has been observed in similar factorial design where pigs were raised in adjacent pens of varying stocking density that shared an air supply (Funk et al. 2007). We did find that microbial communities in single-cage chickens started out similar, but diverged from communities of chickens raised in six and 34-bird cages. A study that placed high- and low-density flocks in isolators also found that high density changed the composition and abundance of the bacterial community by the 3rd week of age, and had a negative impact on broiler growth (Guardia et al. 2011).

Our genotypic data revealed an increase in genotype diversity and evenness, particularly after the first sampling time point. This hints at the importance of strain turnover over the life of the chicken. Samples were less dissimilar between days 19-21 and

days 38, and differed across experiments, suggesting that the community composition did not converge to some stable makeup that was acquired from the coop environment. A similar instability of resistance phenotype and gut genotypes over the life course of the host has been observed in chickens (Bonnet et al. 2009b) and calves (Hoyle et al. 2005), and is most likely part of the normal physiological changes in the animal gut, particularly as microbial communities get established in the first days of life.

Our study has several limitations: first, the experimental system as not closed to the outside environment, potentially confounding comparisons due to influence of external factors: birds in our experimental groups shared an air supply, and other chickens were being raised in the facility. Second, we were limited by the use of culture-based methods, which have known shortcomings (Ovreas 2000). The scope of our study is thus limited only to the dynamics of *E. coli.* Substantially more insight into microbial communities would be gained if we performed bacterial counts to estimate population sizes, or if metagenomic methods were used to look at the abundances of multiple taxa. Finally, our objectives were to look at the population dynamics of the *E. coli* community and relevant resistance phenotypes and genes. We cannot distinguish between the amplification of resistant clones and the emergence of new resistance mechanisms. Despite these limitations, our work is still the first to our knowledge to apply this factorial design of antibiotic treatment and stock density in poultry, and also benefits from the use of population biology approaches to define antimicrobial resistance and study its dynamics.

In conclusion, the commensal microbiota of commercially raised broilers has high levels of antimicrobial resistance and resistance genes, which are likely to persist as birds are slaughtered and distributed. At the level of individual flocks and farming operation, management practices such as antimicrobial regimens and stocking densities have a limited

impact on the ecology of drug-resistant *E coli.* External factors and physiological processes play a larger role in shaping the dynamics of antimicrobial resistance.

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SUPPLEMENTARY MATERIAL

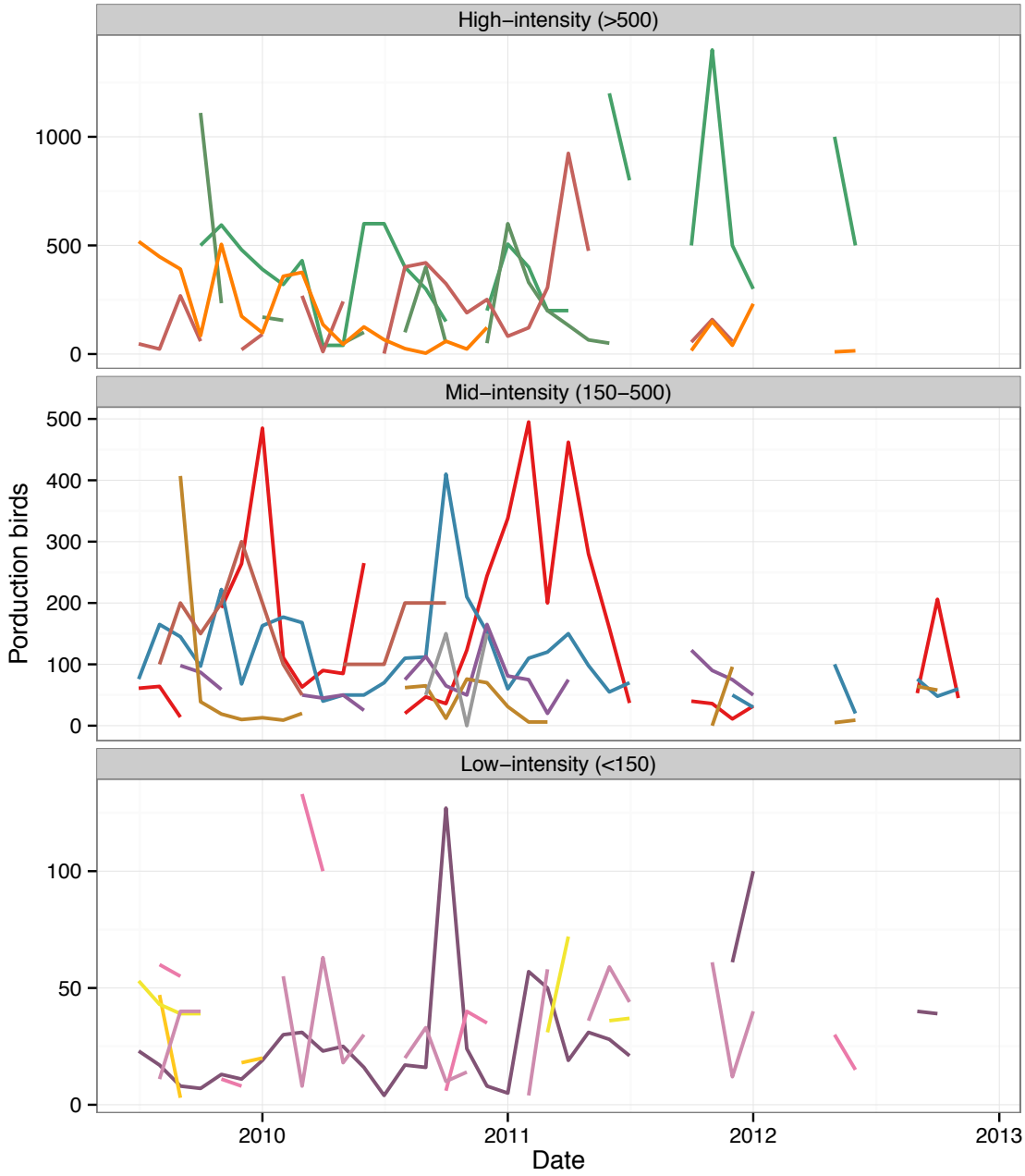
Supplementary table 1: **Chemical analysis of feed samples of the most commonly sold brand of feed (Nutril) purchased at a veterinary store in the large regional center.**

Samples were tested for the presence of lincomycin, virginiamycin, bacitracin, flavomycin, avilomycin, nitrofurantoin, chloramphenicol, tetracycline, tylosin, sulfamethazine, sulfathiazole by mass spectrometry.

Type of animal	**Type of feed**	**Result**
Laying hen	Pre-initial laying hen feed	chloramphenicol
Laying hen	Laying hen feed	tetracycline, virginiamycin
Broiler chicken	Initial feed	virginiamycin
Broiler chicken	Initial feed	chloramphenicol, virginiamycin
Broiler chicken	Fattening/finishing feed	virginiamycin
Broiler chicken	Fattening/finishing feed	chloramphenicol, lincomycin
Any	Ground corn	tetracycline

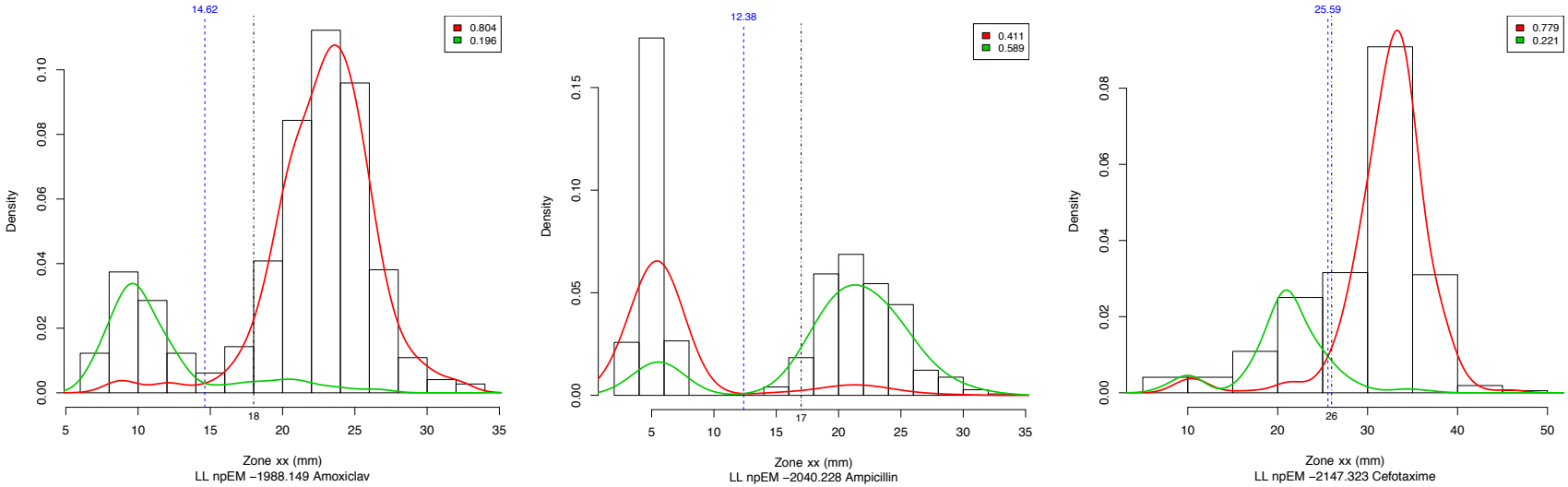
Supplementary figure 1: Monthly census of production birds (broilers and laying hens) in 15 study sites

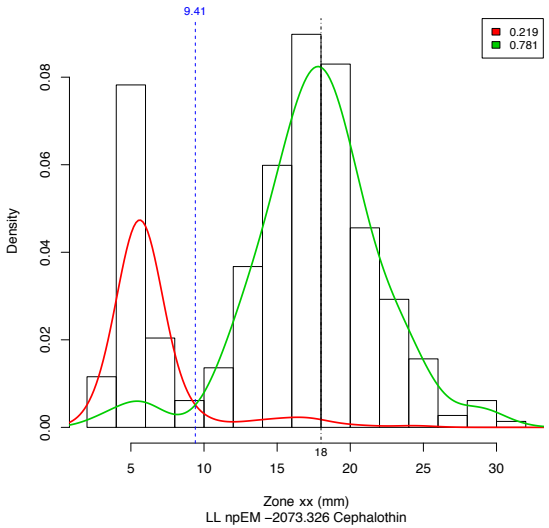
Sites split into groups based on maximum recorded number of birds at any sampling visit. Discontinuity in the lines represents missing data not collected due to logistical limitations.



Supplementary figure 2: Histograms of inhibition zones for production bird E. coli isolates and the fitted two-component mixture models

The proportion of isolates estimated to correspond to each of the two distributions is shown in the legend. We used expected maximum (EM) algorithms for repeated measurement data to account for multiple isolates cultured from each sample. We compared parametric (parEM), semi-parametric (spEM) and non-parametric (npEM) expectations. The best-fit model was selected based on the log-likelihood statistic, shown at the bottom of the graph. Custom breakpoints were set where the density estimates for the fitted distributions intersect. That number is shown in blue. The black dotted line shows the official CLSI susceptibility breakpoint.

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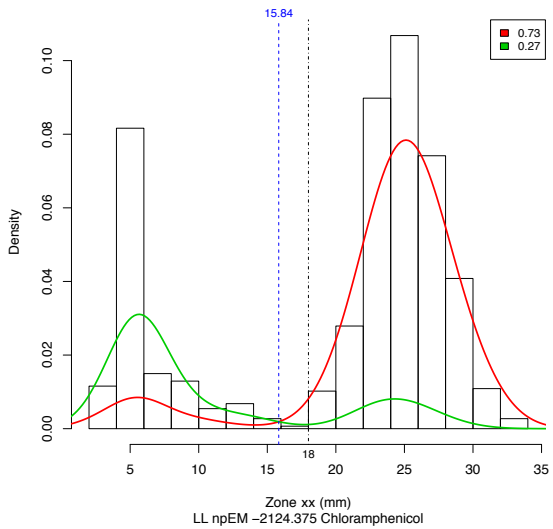
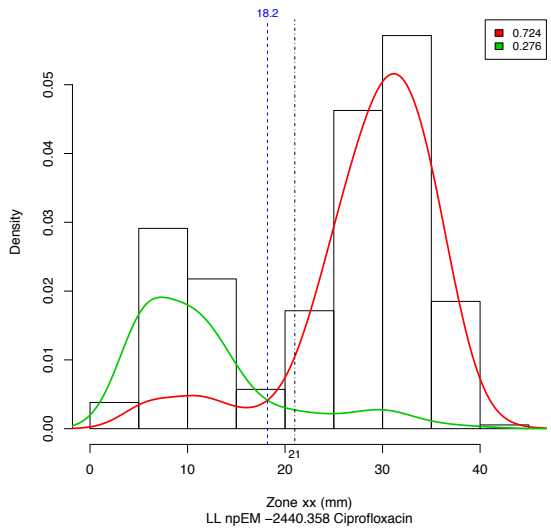
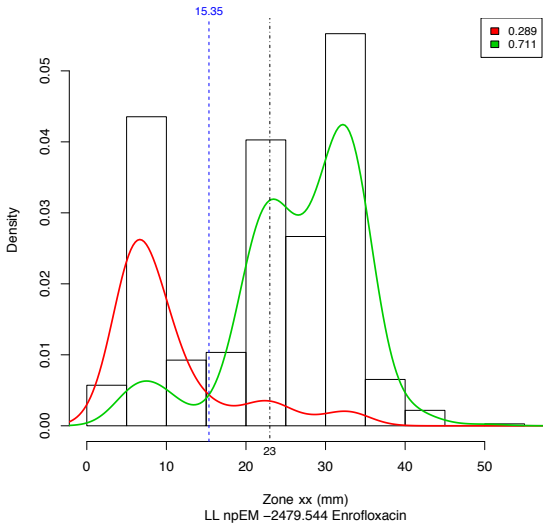
0.219
0.781
Density
Zone xx (mm)
LL npEM –2073.326 Cephalothin


15.84
0.73
0.27
Density
Zone xx (mm)
LL npEM –2124.375 Chloramphenicol


18.2
0.724
0.276
Density
Zone xx (mm)
LL npEM –2440.358 Ciprofloxacin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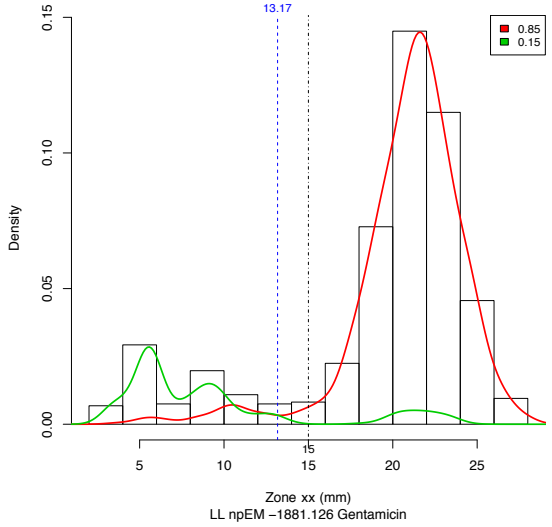
0.289
0.711
Density
Zone xx (mm)
LL npEM –2479.544 Enrofloxacin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0.85
0.15
Density
Zone xx (mm)
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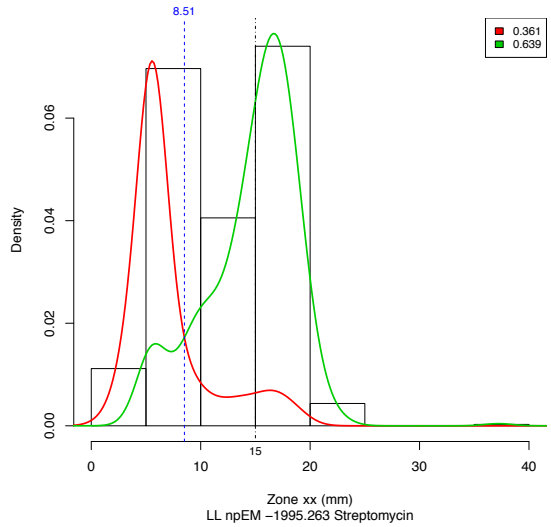
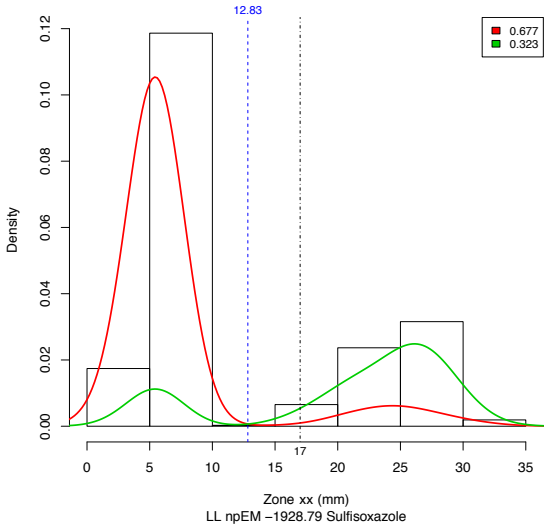
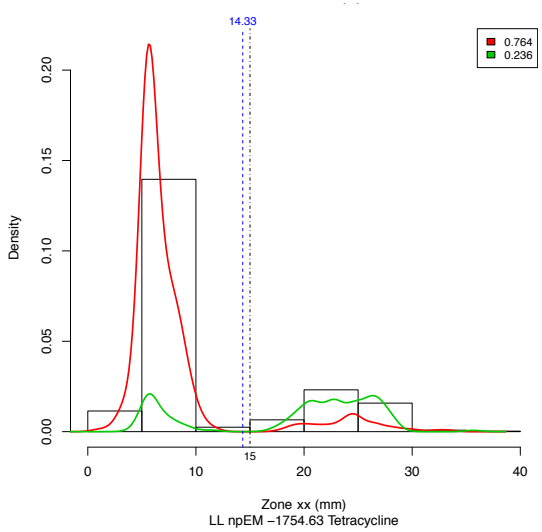
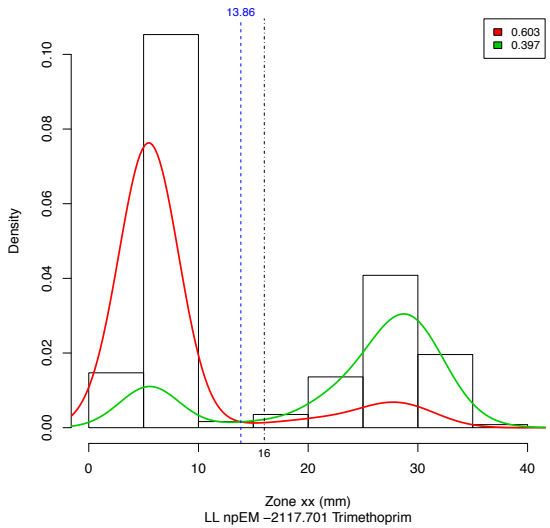

8.51
0.361
0.639
Density
Zone xx (mm)
LL npEM –1995.263 Streptomycin

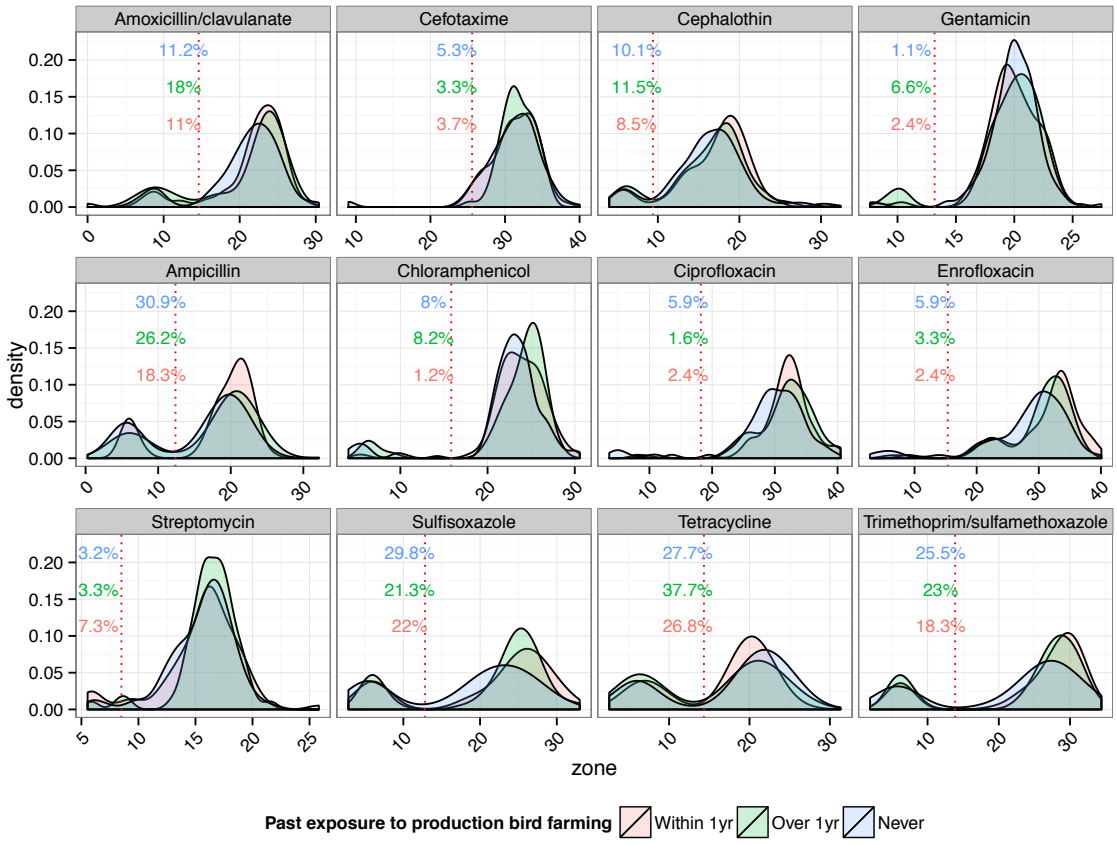

12.83
17
0.677
0.323
Density
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0
5
10
15
20
25
30
35
Zone xx (mm)
LL npEM –1928.79 Sulfisoxazole


14.33
15
0.764
0.236
Density
0.00
0.05
0.10
0.15
0.20
0
10
20
30
40
Zone xx (mm)
LL npEM –1754.63 Tetracycline


13.86
16
0.603
0.397
Density
0.00
0.02
0.04
0.06
0.08
0.10
0
10
20
30
40
Zone xx (mm)
LL npEM –2117.701 Trimethoprim

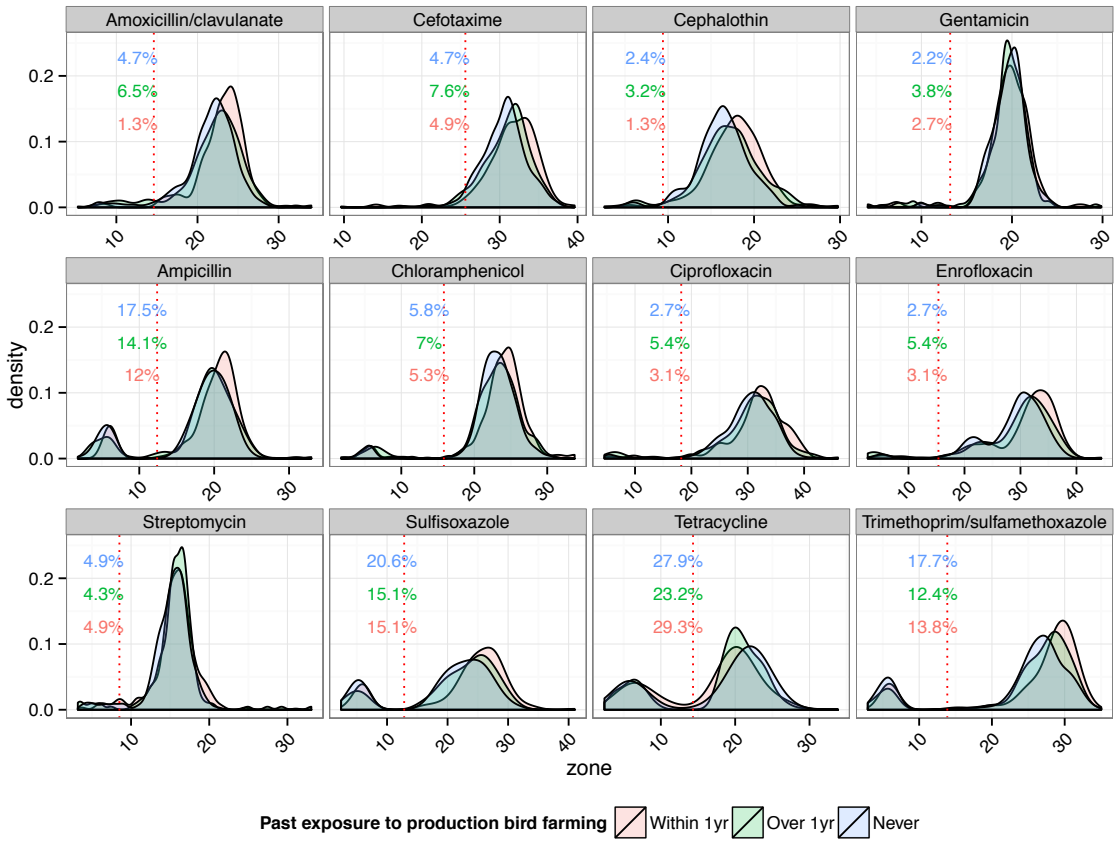
Supplementary figure 3**:** Kernel density estimates of inhibition zone profiles and categorical interpretation of susceptibility tests for *E. coli* isolates from household drinking water samples, by farming history of household

Based on survey data, households were divided into those that had farmed broiler chickens or laying hens ('production birds') within the past year (*N* = 82 isolates from 38 samples), had farmed production birds over one year before the sampling event (*N* = 61 isolates from 28 samples), and those that had never farmed production birds (*N* = 188 isolates from 76 samples). Colored numbers show the percentages of resistant isolates according to the custom susceptibility breakpoint, where the color corresponds to the exposure. There were no significant differences between groups (*P* >0.05 by GLMM-ANOVA and GLMM-logit)



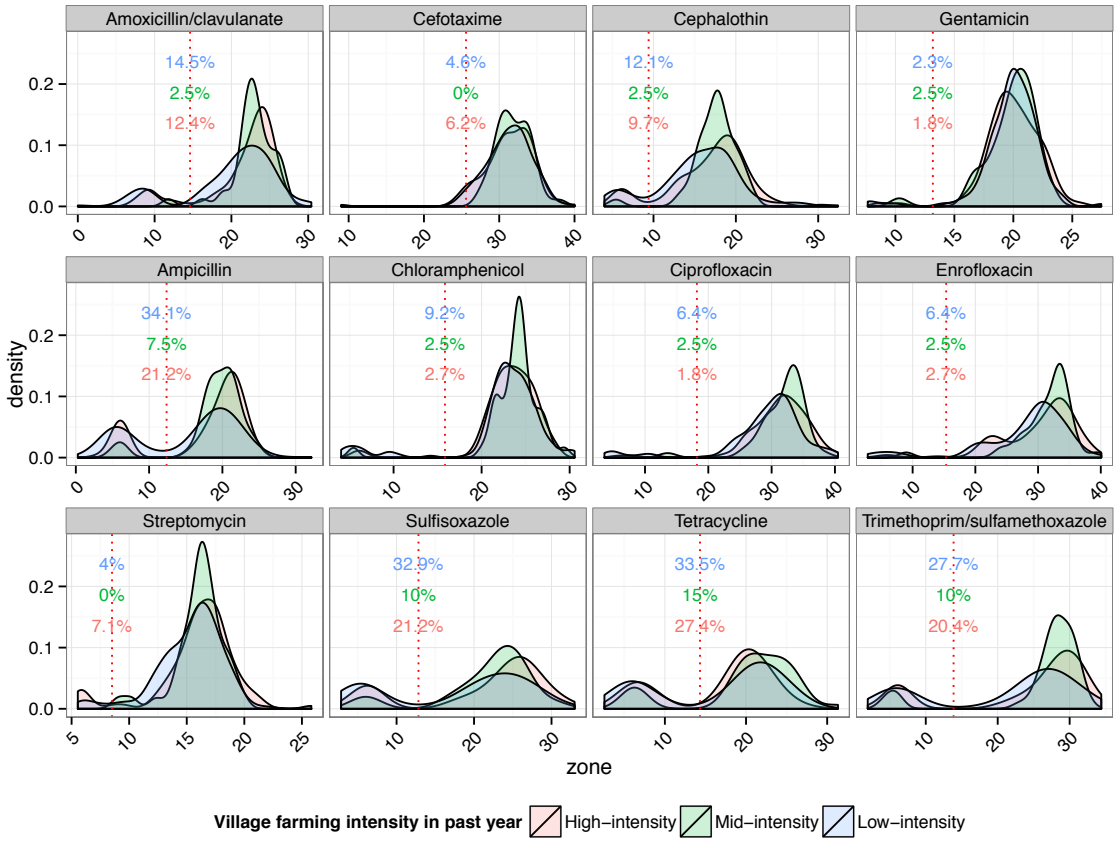
Supplementary figure 4: Kernel density estimates of inhibition zone profiles and categorical interpretation of susceptibility tests for *E. coli* isolates from household soil samples, by farming history of household

Based on survey data, households were divided into those that had farmed broiler chickens or laying hens ('production birds') within the past year (*N* = 225 isolates from 71 samples), had farmed production birds over one year before the sampling event (*N* = 185 isolates from 58 samples), and those that had never farmed production birds (*N* = 451 isolates from 131 samples). Colored numbers show the percentages of resistant isolates according to the custom susceptibility breakpoint, where the color corresponds to the exposure. There were significant differences between groups for amoxicillin/clavulanate (P<0.01 by GLMM-ANOVA), ampicillin (P =0.04), cephalothin (P<0.01), sulfisoxazole (P <0.01) and trimethoprim/sulfamethoxazole (0.03).



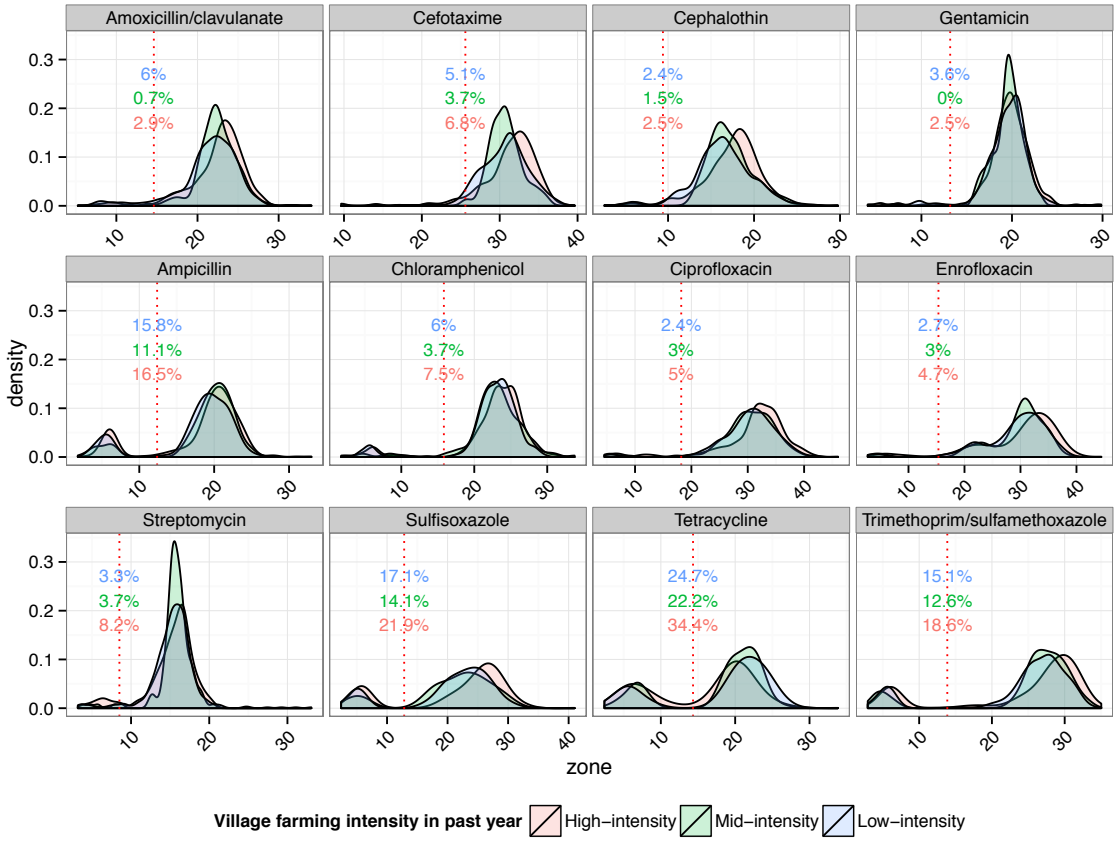
Supplementary figure 5: Kernel density estimates of inhibition zone profiles and categorical interpretation of susceptibility tests for *E. coli* isolates from household drinking water samples, by intensity of farming in the year prior to sampling

Villages were split into high-intensity (>400 birds; *N* = 113 isolates from 55 samples), mid-intensity (100-400 birds; *N* = 40 isolates from 19 samples) and low-intensity (<100 birdss; *N* = 173 isolates from 70 samples) based on maximum recorded number of birds in the one year preceding the sampling visit. There were no significant differences between groups with the exception of ampicillin (*P* =0. 008 by GLMM-logit) and sulfisoxazole (*P* = 0.01), in which cases samples from villages without poultry farming in the past year had higher resistance.



Supplementary figure 6: Kernel density estimates of inhibition zone profiles and categorical interpretation of susceptibility tests for *E. coli* isolates from household soil samples, by intensity of farming in the year prior to sampling

Villages were characterized as high-intensity (>400 birds; *N* = 279 isolates from 90 samples), mid-intensity (100-400 birds; *N* = 135 isolates from 44 samples) and low-intensity (<100 birds; *N* = 449 isolates from 131 samples) based on maximum recorded number of birds in the one year preceding the sampling visit. There were no significant differences between groups with the exception of amoxicillin/clavulanate (*P* =0.03 by GLMM-ANOVA), gentamicin (*P* < 0.01 by GLMM-logit) and tetracycline (*P* < 0.01 by GLMM-ANOVA), in which cases samples from villages with mid-intensity poultry farming in the past year tended to be more resistant than those from high- or low-intensity villages.



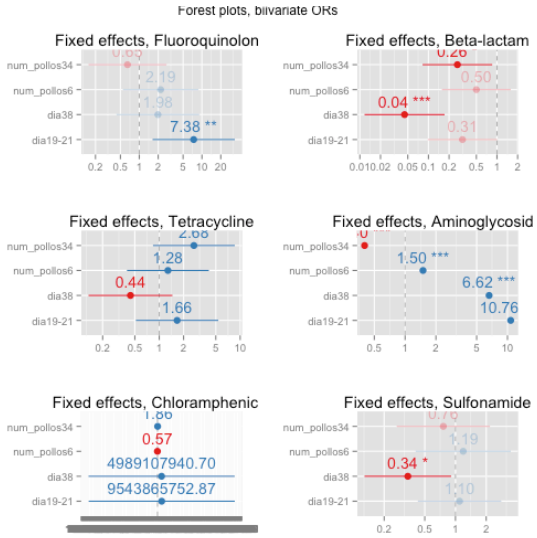
Supplementary table 2: oligonucleotide primers used in the amplification of resistance genes

Targeted genetic marker	Primer type	Sequence (5' – 3')	Size (bp)	Reference or GenBank Accession No.
int1	Forward	GGAATGGCCGAGCAGATCCT	881	Cocchi *et al*.(2007)
	Reverse	CTGCGTTCGGTCAAGGTTCT		
qnrB	Forward			
	Reverse			
tetA	Forward	TTGGCATTCTGCATTCACTC	475	AJ419171
	Reverse	GTATAGCTTGCCGGAAGTCG		
tetB	Forward	CAGTGCTGTTGTTGTCATTAA	571	EF646764
	Reverse	GCTTGGAATACTGAGTGTAA		

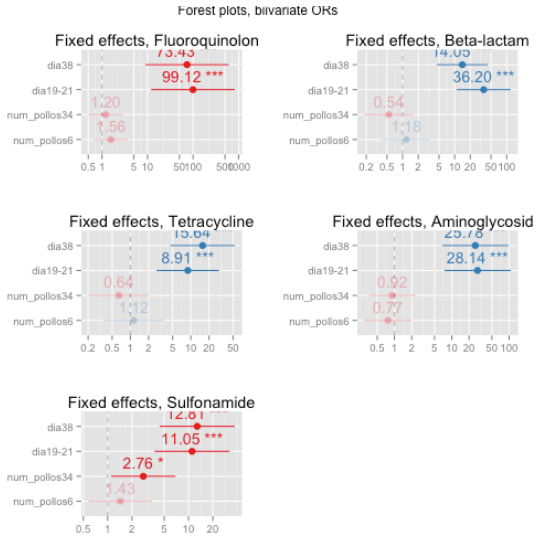
Supplementary figure 7: forest plots of bivariate models for the proportion of resistant isolates over time and across stocking density groups, by trial

Forest plot showing Odds Ratios and 95% Confidence Intervals from Generalized Linear Mixed Effect Models fitted by maximizing a Restricted Maximum Likelihood Criterion with nested random effects for sample and chicken. Colors reflect the direction of the effect. Type 3 like *p*-values calculated using an approximation for degrees-of-freedom. Estimates with *P*-values <0.05 are in bold, and are annotated with stars corresponding to *P*<0.05(*), *P*<0.01(**), *P*<0.001 (***). Intercept estimates not shown.

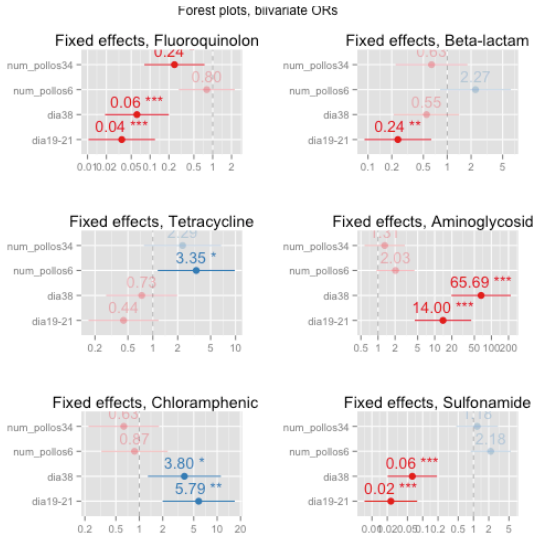
A) Trial 1a (tetracycline/fresh litter)



B) Trial 2a (enrofloxacin/fresh litter)



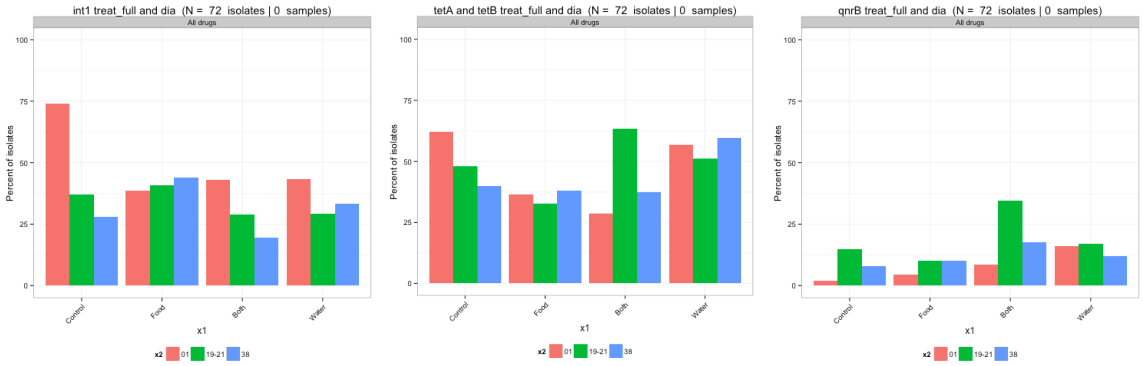
C) Trial 2b (enrofloxacin/used litter)



Supplementary figure 9: proportion of resistant isolates over time and across stocking density groups, by trial

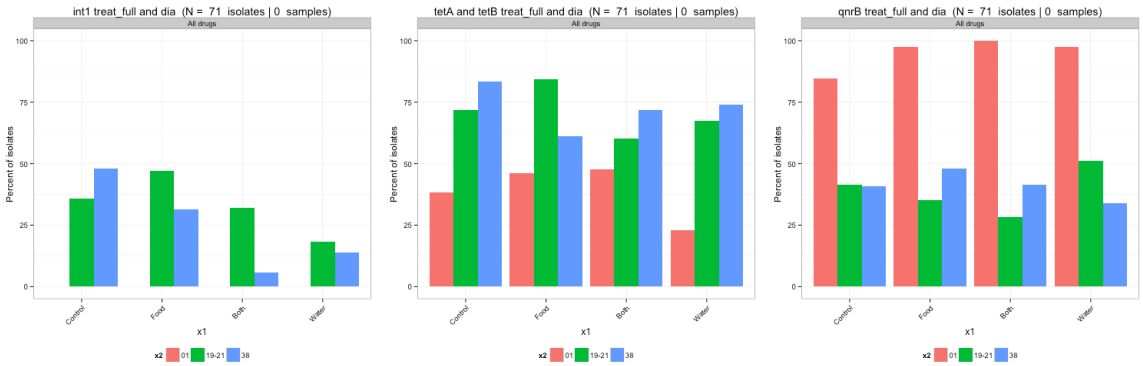
A) Trial 1a (tetracycline/fresh litter)

int1 tetAB qnrb



B) Trial 2a (enrofloxacin/fresh litter)

int1 tetAB qnrb



C) Trial 2b (enrofloxacin/used litter)

int1 tetAB qnrb