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Lower Prevalence of Antibiotic-resistant Enterococci On U.S. Conventional Poultry Farms That Transitioned to Organic Practices

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Abbreviations:

AGP	Antimicrobial growth promoter
ARIS	Automated reading and incubation system
AST	Antimicrobial susceptibility testing
BHI	Brain heart infusion
CFU	Colony forming unit
CHL	Chloramphenicol
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CONV	Conventional
DAP	Daptomycin
EA	Enterococcosel agar
EPA	U.S. Environmental Protection Agency
ERY	Erythromycin

FLA	Flavomycin
GEN	Gentamicin
GLMM	Generalized linear mixed models
GMOs	Genetically modified organisms
KAN	Kanamycin
LIN	Lincomycin
LZE	Linezolid
MDR	Multi-drug resistance
MIC	Minimal inhibitory concentration
NARMS	National Antimicrobial Resistance Monitoring System
NIT	Nitrofurantoin
NOP	National Organic Program
ORG	Organic
PEN	Penicillin
PYR	Pyrrolidonyl arylamidase
STR	Streptomycin
SYN	Quinupristin/dalfopristin
TET	Tetracycline
TIG	Tigecycline
TSA	Tryptic soy agar
TYL	Tylosin
USDA	U.S. Department of Agriculture
VAN	Vancomycin

Abstract:

Background: In U.S. conventional poultry production, antimicrobials are used for therapeutic, prophylactic and non-therapeutic purposes. Researchers have shown that this can select for antibiotic-resistant commensal and pathogenic bacteria on poultry farms and poultry-derived products. However, no U.S. studies have investigated on-farm changes in resistance as conventional poultry farms transition to organic practices and cease using antibiotics.

Objective: To evaluate the prevalence of antibiotic-resistant *Enterococcus* on U.S. conventional poultry farms that transitioned to organic practices.

Methods: Poultry litter, feed, and water samples were collected from 10 conventional and 10 newly organic poultry houses in 2008 and tested for *Enterococcus*. *Enterococcus* (n=259) was identified using the Vitek ®Compact 2 System, and tested for susceptibility to 17 antimicrobials using the Sensititre™ microbroth dilution system. Data were analyzed using SAS v9.2 and statistical associations were derived based on generalized linear mixed models.

Results: Litter, feed and water samples were *Enterococcus*-positive. The percentages of resistant *E. faecalis* and resistant *E. faecium* were significantly lower ($p<0.05$) among isolates from newly organic versus conventional houses for two (erythromycin and tylosin) and five (ciprofloxacin, gentamicin, nitrofurantoin, penicillin and tetracycline) antimicrobials, respectively. Forty-two percent of *E. faecalis* isolates from conventional poultry houses were multi-drug resistant (MDR) (to ≥ 3 antimicrobial classes) compared to 10% of isolates from newly organic houses ($p=0.02$), and 84% of *E. faecium* isolates from conventional houses were MDR compared to 17% of isolates from newly organic poultry houses ($p<0.001$).

Conclusions: Our findings suggest that the voluntary removal of antibiotics from large-scale U.S. poultry farms that transition to organic practices is associated with a lower prevalence of antibiotic-resistant and MDR *Enterococcus*.

Introduction

Antibiotic use in U.S. conventional poultry production poses potential public health concerns with regard to the selection of antibiotic-resistant foodborne bacteria (Institute of Medicine 1998; Levy and Marshall 2004; Silbergeld et al. 2008). In U.S. conventional poultry production, antibiotics are administered for therapeutic, prophylactic and non-therapeutic purposes (Sapkota et al. 2007; Wegener 2003). Some researchers have estimated that antimicrobial use in conventional U.S. poultry production (on a per-bird basis) increased by 307% from 1985 to the late 1990s, with the use of non-therapeutic antimicrobial growth promoters (AGPs) accounting for a significant portion of this use (Mellon et al. 2001).

The use of AGPs in conventional poultry production selects for resistant bacterial populations in the production environment and retail poultry products (Aarestrup et al. 2000a; Hayes et al. 2001; Hayes et al. 2005; Price et al. 2005; Witte 2000). Consequently, the amplification of resistant bacteria in poultry can result in possible increases in the risk of antibiotic-resistant bacterial infections in human populations (Aarestrup et al. 2000a; Hammerum et al. 2010; Levy 1998). Animal-derived antibiotic-resistant bacteria have been shown to spread from animals to humans through direct contact with animals and through the consumption of meat products (Donabedian et al. 2003; van Loo et al. 2007).

These findings are increasingly reported in mainstream news and have become one of the main drivers influencing consumer demand for organic poultry (Oberholtzer et al. 2006) which is perceived to be safer than conventional poultry (Crandall et al. 2009). This consumer demand has spurred increased production of organic poultry, making poultry one of the fastest growing

segments of the U.S. organic products sector (Fanatico et al. 2009; Oberholtzer et al. 2006). Retail sales of organic poultry quadrupled between 2003 and 2006 and reached nearly \$200 million in 2008 (Oberholtzer et al. 2006).

To accommodate increased consumer demand and to profit from the organic poultry niche, some conventional poultry growers are adopting organic practices and transitioning their conventional farms to certified organic poultry farms (Oberholtzer et al. 2006). These transitions—which include cessations in the use of all antibiotics and agrichemicals (Fanatico et al. 2009)—could result in changes in the prevalence of antibiotic-resistant bacteria on newly organic poultry farms and subsequent organic poultry products. European studies suggest that removing the non-therapeutic use of antibiotics from poultry farms can result in statistically significant reductions in antibiotic-resistant bacteria in animals and food products (Aarestrup et al. 2000b; Aarestrup et al. 2001; Emborg et al. 2003; Hammerum et al. 2007; Heuer et al. 2002; Klare et al. 1999). Reductions in human carriage of resistant bacteria also have been documented in association with antibiotic withdrawals in European poultry production (Klare et al. 1999; van den Bogaard et al. 2000).

However, to date, the studies regarding this issue that have been conducted in the U.S. have been largely cross-sectional in nature (Han et al. 2009; Price et al. 2007). To the best of our knowledge, no prospective studies have been conducted in the U.S. to quantify on-farm, temporal changes in antibiotic resistance of food-borne bacteria when antibiotics are removed from U.S. poultry production environments. Voluntary transitions to organic practices among large-scale U.S. poultry producers provide an excellent opportunity to research this issue within

the U.S. Thus, the objective of this study was to prospectively evaluate the prevalence of antibiotic-resistant enterococci on large-scale conventional poultry farms that transitioned to organic practices. Findings from year one of this study are described herein.

Materials and Methods

Study sites

All of the poultry farms participating in this study were located in the Mid-Atlantic United States. Two types of poultry farms were included: large-scale conventional broiler farms that were maintaining conventional practices and using antibiotics (n=5), and large-scale (previously conventional) broiler farms that had just received organic certification and were producing their first flock of certified organic broilers (n=5). All participating farms were operating under the guidance of one feed mill that produced both conventional and certified organic poultry feed. Two individual poultry houses from each farm were included in the study for a total of 20 poultry houses. Characteristics of the conventional and newly organic poultry houses are summarized in Table 1.

All of the newly organic poultry houses were certified organic by a state agency accredited by the U.S. National Organic Program (NOP), which promulgates federal organic standards. An overview of common interpretations of the NOP standards that must be met before a poultry farm can be certified organic is provided in Appendix 1.

The specific antimicrobials that were used in feed in the conventional poultry houses were: bacitracin (50g/ton), virginiamycin (15g/ton), roxarsone (45.35g/ton), salinomycin (60g/ton), nicarbazine (0.0125%) and decoquinate (27.2g/ton). In addition, gentamicin was used at the hatcheries that supplied chicks to conventional poultry houses, and bacitracin, virginiamycin, roxarsone and salinomycin were used at the breeder facilities that supplied the initial eggs to the hatcheries.

Sample collection

From March to June 2008, poultry litter, water and feed samples were aseptically collected--in sterile Whirl-Pak® collection bags (Nasco, Fort Atkinson, WI)--from all conventional and newly organic poultry houses. Litter samples (500g) from the top 1 to 5 cm of litter were collected from 3 randomly selected areas. Two water samples (500mL) were retrieved from 1) raw source water before filtration or UV treatment; and 2) finished water present in the waterlines. One poultry feed sample (300g) was collected from the central feed hopper within each house. All poultry litter, water and feed samples were shipped overnight at 4°C and processed within 24 hr.

Isolation

Poultry litter and feed samples were enriched in a 1:10 weight to volume dilution of 100 mL of Enterococcosel Broth (Becton Dickinson & Co., Franklin Lakes, NJ) for 24 hr at 41°C. Positive and negative control broths were included for quality control and quality assurance. After 24 hr, 10 µL of the enrichment culture was streaked onto Enterococcosel Agar (EA) (Becton Dickinson & Co., Franklin Lakes, NJ) and incubated overnight at 41°C. Presumptive

colonies of *Enterococcus* spp. ranged in appearance from brown to black with a brown-black precipitate on EA agar. Three presumptive *Enterococcus* colonies from each litter and feed sample were streaked onto separate Brain Heart Infusion (BHI) agar plates for purification and incubated at 41°C for 24 hr. A colony was collected from each BHI purification plate and archived at -80°C in Brucella broth with 20% glycerol.

Isolation of *Enterococcus* spp. from water samples was performed in accordance with U.S. Environmental Protection Agency (EPA) Method 1106.1 (U.S.EPA 2006). Briefly, 10-fold dilutions of each water sample were prepared in phosphate buffered saline (U.S.EPA 2006), and 10 mL of each dilution was filtered through a 0.45µm, 47 mm mixed cellulose ester filter (Millipore, Billerica, MA). Each filter was placed on a 60 mm plate containing EA, inverted and incubated at 41°C for 24 hr. Resulting colonies typical of *Enterococcus* spp. were considered presumptive *Enterococcus* spp. Out of the recovered presumptive *Enterococcus* spp., three isolates per water sample were purified on BHI and archived in Brucella broth with 20% glycerol at -80°C. Positive (*Enterococcus faecalis* ATCC 29212) and negative (*Escherichia coli* ATCC 25922) controls were used throughout the isolation process.

Identification

All presumptive *Enterococcus* spp. were streaked from archived stocks onto tryptic soy agar (TSA) amended with 5% sheep's blood and incubated at 41°C for 24 hr. Presumptive identification of *Enterococcus* spp. was done by gram staining and testing for catalase production and pyrrolidonyl arylamidase (PYR) activity. All gram-positive, catalase negative, and PYR positive isolates were confirmed and identified to the species-level using the automated

biochemical identification Vitek®2 Compact System (BioMérieux Inc., Hazelwood, MO) in accordance with the manufacturer's specifications.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) was performed on all confirmed *Enterococcus* isolates (n=259) by microbroth dilution using the Sensititre™ system (Trek Diagnostic Systems, Westlake, Ohio) according to the manufacturer's directions. Briefly, colonies from pure 18-24 hr cultures were transferred to tubes of sterile Sensititre demineralized water (Trek Diagnostic Systems, Westlake, Ohio) to achieve a turbidity equivalent to a 0.5 McFarland standard. Then, 50µL of each suspension was added to sterile Sensititre, cation-adjusted, Mueller Hinton broth (Trek Diagnostic Systems, Westlake, Ohio), and 50µL of the broth solution was then dispensed into microtitre, gram-positive 96-well plates embedded with 17 test antimicrobials (National Antimicrobial Resistance Monitoring System (NARMS) *Enterococcus* Plate Format; Trek Diagnostic Systems, Westlake, Ohio). Plates were then incubated in the Automated Reading and Incubation System (ARIS) at 37°C for 18 ± 1hr. The first 100 plates were read both manually and via the ARIS system for quality assurance comparisons of minimal inhibitory concentration (MIC) determinations. Having determined consistency between the two methods, subsequent samples were read by the ARIS exclusively.

Clinical and Laboratory Standards Institute (CLSI) interpretive criteria for microbroth dilution methods (CLSI 2008) were used to evaluate resulting MICs where breakpoints were available, except for quinupristin/dalfopristin for which the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoint was used (EUCAST 2011).

Otherwise, provisional breakpoints used by NARMS were utilized (U.S.FDA 2009). The following specific antimicrobials and resistance breakpoints were used: chloramphenicol (CHL, ≥ 32), ciprofloxacin (CIP, ≥ 4), daptomycin (DAP, no interpretive criteria available), erythromycin (ERY, ≥ 8), flavomycin (FLA, ≥ 32), gentamicin (GEN, > 500), kanamycin (KAN, ≥ 1024), lincomycin (LIN, ≥ 8), linezolid (LZE, ≥ 8), nitrofurantoin (NIT, ≥ 128), penicillin (PEN, ≥ 16), streptomycin (STR, > 1000), quinupristin/dalfopristin (SYN, ≥ 8), tetracycline (TET, ≥ 16), tigecycline (TIG, no interpretive criteria available), tylosin (TYL, ≥ 32), and vancomycin (VAN, ≥ 32). Multi-drug resistance (MDR) was defined as acquired resistance to three or more antimicrobial classes. *Enterococcus faecalis* ATCC 29212, *E. faecalis* ATCC 51299, *Staphylococcus aureus* ATCC 29213, and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains.

Statistical analysis

The generalized linear mixed models (GLMM) method was used to evaluate associations between the prevalence of antibiotic-resistant *Enterococcus* spp. and poultry production type (conventional or newly organic). The GLMM method was used to account for the clustered nature of the study design which made it necessary to adjust for intra-poultry house and intra-poultry farm variability. Firth's bias correction method was used when zero counts occurred for one group (Heinze and Pühr 2010). All statistical analyses were performed using SAS v9.2 (SAS Institute Inc, 2008).

Results

Prevalence of Enterococcus spp.

Enterococcus spp. was isolated from 100% of all conventional and newly organic poultry houses. Poultry litter was the principal environmental media for the recovery of *Enterococcus* spp. from both farm types with 100% of all litter samples testing positive; however, these microorganisms were also recovered from water and feed samples (Table 2).

Overall, 46% of *Enterococcus* spp. were identified as *E. faecalis* and 43% as *E. faecium*. *E. durans*, *E. gallinarum*, and *E. hirae* were also isolated from both types of houses in several types of environmental media (Table 2). There were no significant differences in species prevalence between farm types.

MICs

MIC ranges, MIC50s and MIC90s for *E. faecalis* and *E. faecium* recovered from the poultry houses are shown in Table 3. Fifty-three percent of MIC ranges (8/15 antibiotics, excluding lincomycin and quinupristin/dalfopristin due to inherent resistance) among *E. faecalis* differed depending on farm type, while 56% of MIC ranges (9/16 antibiotics, excluding flavomycin due to inherent resistance) among *E. faecium* differed depending on farm type (Table 3). Some MIC ranges differed depending on species (Table 3). Similarly, some differences in MIC50s and MIC90s were also observed between isolates recovered from different farm types, and between species (Table 3).

Acquired Antibiotic Resistance

Among *E. faecalis* isolates, acquired antibiotic resistance against the following nine antimicrobials was lower among *E. faecalis* from newly organic versus conventional poultry houses: chloramphenicol, erythromycin, flavomycin, gentamicin, kanamycin, nitrofurantoin, penicillin, streptomycin, and tylosin (Figure 1, Panel A). The differences in percent resistance were statistically significant for erythromycin ($p=0.004$) and tylosin ($p=0.004$) (Figure 1, Panel A).

Among *E. faecalis*, acquired resistance to chloramphenicol, gentamicin and penicillin was observed only among isolates recovered from conventional poultry houses (Figure 1, Panel A). Gentamicin is one of the antibiotics used at the hatcheries that supplied chicks to the conventional poultry houses. No resistance to linezolid or vancomycin was observed among any of the *E. faecalis* recovered from conventional or newly organic poultry houses (Figure 1, Panel A). The absence of vancomycin resistance is most likely attributed to the fact that glycopeptides have never been approved for use in U.S. animal agriculture.

Among *E. faecium* isolates, acquired antibiotic resistance against the following 11 antimicrobials was lower among *E. faecium* from newly organic versus conventional poultry houses: ciprofloxacin, erythromycin, gentamicin, kanamycin, lincomycin, nitrofurantoin, penicillin, streptomycin, quinupristin/dalfopristin, tetracycline and tylosin (Figure 1, Panel B). The differences in percent resistance were statistically significant for ciprofloxacin ($p=0.01$), gentamicin ($p=0.047$), nitrofurantoin ($p=0.02$), penicillin ($p<0.001$) and tetracycline ($p<0.001$) (Figure 1, Panel B).

Among *E. faecium*, acquired resistance to gentamicin and tylosin was observed only among isolates recovered from conventional poultry houses (Figure 1, Panel B). No resistance to chloramphenicol, linezolid, or vancomycin was observed among any of the *E. faecium* recovered from conventional or organic poultry houses (Figure 1, Panel B).

Sources of Antibiotic-resistant Bacteria

The majority of antibiotic-resistant *E. faecalis* were isolated from poultry litter samples (Table 4). *E. faecalis* isolated from conventional feed samples also expressed acquired resistance to 8 antimicrobials (CHL, ERY, FLA, KAN, NIT, STR, TET, TYL), indicating that conventional poultry feed could be a potential source of exposure to antibiotic-resistant *E. faecalis* among broilers (Table 4). No resistant *E. faecalis* were isolated from organic poultry feed or source water or waterline samples retrieved from either conventional or newly organic poultry houses.

The majority of antibiotic-resistant *E. faecium* also were isolated from poultry litter samples (Table 4). Antibiotic-resistant *E. faecium* were also recovered from feed and waterline samples from conventional poultry houses, and feed, source water and waterline samples from newly organic poultry houses (Table 4). Conventional feed was contaminated with *E. faecium* that expressed acquired resistance to 10 antimicrobials (CIP, ERY, KAN, LIN, NIT, PEN, STR, SYN, TET, TYL), while organic feed was contaminated with *E. faecium* that expressed acquired resistance to 6 antimicrobials (ERY, KAN, LIN, NIT, SYN, TET) (Table 4). No conventional source water samples were contaminated with resistant *E. faecium*, while one organic source

water sample was contaminated with one lincomycin-resistant *E. faecium*. Conventional waterline samples were contaminated with *E. faecium* that expressed acquired resistance to 11 antimicrobials (CIP, ERY, GEN, KAN, LIN, NIT, PEN, STR, SYN, TET, TYL), while organic waterline samples were not contaminated with antibiotic-resistant *E. faecium* (Table 4). The differences in waterline contamination between house types could be attributed to the fact that conventional poultry houses, in general, were older than newly organic houses (Table 1), allowing more time for contamination to occur.

Acquired Multi-drug Resistance

The percentage of MDR *E. faecalis* was statistically significantly lower among isolates from newly organic poultry houses compared to isolates from conventional houses (10% vs 42%, $p=0.02$; Figure 2). The percentage of MDR *E. faecium* also was statistically significantly lower among isolates from newly organic poultry houses compared to isolates from conventional houses (17% vs 84%, $p<0.001$; Figure 2). Predominant MDR patterns are shown in Table 5.

The mode number of antibiotics that *E. faecalis* expressed acquired resistance against was 1 and 3, among isolates from newly organic houses and conventional houses, respectively (Figure 3, Panel A). The mode number of antibiotics that *E. faecium* expressed acquired resistance against was 1 and 4, among isolates from newly organic houses and conventional houses, respectively (Figure 3, Panel B). These findings show that newly organic poultry houses are characterized by individual *E. faecalis* and *E. faecium* isolates that express resistance to fewer numbers of antibiotics compared to their conventional counterparts.

Discussion

In this study, we observed a significantly lower prevalence of antibiotic-resistant and MDR *E. faecalis* and *E. faecium* on large-scale poultry farms that had just transitioned to organic practices compared to large-scale poultry farms that were maintaining conventional practices. To our knowledge, these are the first U.S. data to show immediate, on-farm changes in antibiotic resistance when antimicrobials are voluntarily withdrawn from large-scale U.S. poultry production.

These findings are in agreement with earlier European and Asian studies that have documented reductions in antibiotic-resistant *Enterococcus* spp. following governmental bans and/or voluntary withdrawals of the use of antibiotics in animal production (Aarestrup et al. 2001; Lauderdale et al. 2007). Using data from the Danish program for surveillance of antimicrobial resistance in bacteria recovered from animals, foods and humans (DANMAP), Aarestrup et al. (2001) reported significant decreases in the percentages of *E. faecalis* and *E. faecium* resistant to avilamycin, erythromycin, avoparcin, and virginiamycin, four antibiotics banned by the Danish government for use as AGPs in the late 1990s (Aarestrup et al. 2001). For example, from 1997 to 2000 the percentage of erythromycin-resistant *E. faecium* isolated from broilers decreased from 76.3% to 12.7%, and the percentage of virginiamycin-resistant *E. faecium* isolated from broilers decreased from 66.2% to 33.9% (Aarestrup et al. 2001). In the present study, we observed that the prevalence of *E. faecium* resistant to erythromycin was 13% and 10% among isolates from conventional and newly organic farms, respectively, while the prevalence of *E. faecium* resistant to quinupristin/dalfopristin (a virginiamycin analogue) was

20% and 7% among isolates from conventional and newly organic farms, respectively (Figure 1, Panel B).

Reductions in percent resistance to erythromycin and other antibiotics observed among *Enterococcus* spp. from newly organic poultry farms in the present study may not be as dramatic as those observed in the Aarestrup et al. (2001) study and other European reports because poultry houses in the present study were sampled during the production of the first flock of certified organic broilers. While these poultry houses underwent extensive and comprehensive cleaning events before they could be certified as organic, reservoirs of resistant bacteria may have remained in the packed dirt floor and on fomites within the houses, contributing to persistent low-levels of antibiotic-resistant enterococci in newly organic houses. Similarly, Heuer et al. (2002) demonstrated that vancomycin-resistant enterococci can persist in broiler flocks for more than 5 years after antibiotic selective pressures are removed from the production environment.

Two additional factors likely play significant roles in the persistence of low rates of antibiotic-resistant enterococci observed in newly organic poultry houses in this study. First of all, U.S. organic certification standards, promulgated through the NOP, apply starting on day 1 of a chick's life (Fanatico et al. 2009). No organic certification standards need to be met prior to the first day of life. Thus, some breeder facilities that supply eggs to hatcheries, and hatcheries that ultimately produce "organic" chicks, do not have to meet any organic standards, and can therefore utilize antibiotics among breeder stocks and inject antibiotics into eggs. These

practices can result in exposures to antibiotics among “organic” broilers prior to the first day of life.

In addition to these exposures, organic broilers can be exposed to antibiotic-resistant bacteria through feed and water. Organic poultry feed is required by the NOP to be free of antibiotics, slaughter byproducts and genetically modified organisms (Fanatico et al. 2009). However, our data show that contamination of organic feed with antibiotic-resistant bacteria can occur (Table 4). The question remains as to whether feed is contaminated at the feed mill, during transport, and/or during storage at poultry houses via bioaerosols, insects, rodents or other factors. Beyond feed, we observed that one source water sample from newly organic houses was contaminated with one lincomycin-resistant *E. faecium* (Table 4) and one waterline sample from organic houses was contaminated with one MDR *E. gallinarum* (data not shown).

Encouraging findings from this study were our observations that the percentages of MDR *E. faecalis* and MDR *E. faecium* were significantly lower on newly organic poultry farms compared to farms that were maintaining conventional practices. *E. faecalis* recovered from newly organic and conventional farms expressed acquired resistance against a mode number of 1 and 3 antibiotics, respectively, and *E. faecium* from newly organic and conventional farms were resistant against a mode number of 1 and 4 antibiotics, respectively. These data are in agreement with a recent study by Miranda et al. (2007) that showed that rates of MDR *Enterococcus* spp. were significantly lower among isolates recovered from organic chicken and turkey products compared to conventional products (Miranda et al. 2007).

As with all field-based studies, this study had several limitations. As discussed above, we could not control for the fact that organic broilers may have been exposed to antibiotics prior to the first day of life. This could have influenced the rates of antibiotic resistance observed among *Enterococcus* spp. recovered from newly organic poultry houses; however, because we could not include a control farm that produced chicks that were known to have never been exposed to antibiotics, we could not estimate the contributions of these potential exposures to observed resistance rates. The study is also limited in terms of geographical location. All poultry farms included in this study are located in the Mid-Atlantic U.S., and under the advisement of one feed mill. Thus, it is unclear whether our results are generalizable across the U.S. and across the various large-scale contract growers that dominate the U.S. poultry industry. Larger scale studies based in varying geographical areas at farms managed by different companies are necessary. Finally, the study is limited by the fact that separate conventional poultry farms served as control farms for the newly organic poultry farms. While it would have been preferable to also utilize the newly organic poultry farms prior to their conversion from conventional to organic practices, this was not possible.

Conclusions

In summary, this study provides the first on-farm U.S. data describing the impacts of eliminating antibiotics from large-scale U.S. poultry production on rates of antibiotic-resistant enterococci. The findings support the hypothesis that removing antibiotic use from large-scale U.S. poultry farms transitioning to organic practices can result in immediate and statistically significant reductions in on-farm antibiotic resistance.

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Appendix 1: Overview of common interpretations of U.S. National Organic Program (NOP) standards for broiler production (Fanatico et al. 2009;USDA AMS 2010).

Common Interpretations of NOP Standards:

Producer must create and implement an organic system plan and manure management plan.

Broilers must be produced under continuous organic management starting “no later than the second day of life.”

All feed components must be organically produced and contain no antibiotics, other animal drugs, slaughter byproducts, or genetically modified organisms (GMOs).

No antibiotics may be used for animal treatment.

Producer must establish preventative broiler health care practices, and diseases can be prevented with vaccines, biosecurity measures, prebiotics and probiotics.

Maximum stocking densities of broilers is not specified by the NOP, but certifying agencies often require at least 0.14 m² per bird.

An outdoor access area must be provided to ensure access to fresh air, exercise and sunlight.

Clean and dry bedding must be provided in an indoor area.

Sanitizers and cleaners used on the property must be on approved products lists.

Agrichemicals cannot be used on the property.

Table 1: Characteristics of poultry houses at the time of sampling.

Characteristic	Mean (Min,Max)	
	Conventional (n=10)	Organic (n=10)
Months that Farm Practiced Organic Methods	0	1.72(0, 3.6)
Number of Antibiotics Used in Feed	3(2, 4)	0
Number of Antibiotics Used in Water	0.18(0, 1)	0
Age of House (Years)	15.7(3, 30)	8.8(3, 15)
Length of House (Feet)	407(110, 500)	500(500, 500)
Width of House (Feet)	44.5(35, 50)	46.8(44, 48)
Months Since Complete Poultry Litter Change	1.2(1, 1.5)	3.8(1, 12)
Number of Broiler Chicks When Flock Arrived	30,800(30,800, 30,800)	22,608(19,300, 24,000)
Age of Flock (Days)	36(31, 40)	36(29, 41)
Cumulative Mortality Rate (%)	2.51(1.3, 4.3)	4.72(3, 7.5)
Minutes that Broilers Spent Outdoors	0	0

Table 2: *Enterococcus* spp. isolated from litter, feed and water samples collected from conventional and newly organic poultry farms.

Farm Type	<i>Enterococcus</i> Species					
	Number (% of Total Isolates from Farm Type)					
	<i>E. durans</i>	<i>E. faecalis</i>	<i>E. faecium</i>	<i>E. gallinarum</i>	<i>E. hirae</i>	Other
Conventional						
Litter (n=90)	1(<1)	45(34)	42(32)	1(<1)	1(<1)	0(0)
Feed (n=29)	0(0)	10(7)	15(11)	1(<1)	3(2)	0(0)
Source water (n=1)	1(<1)	0(0)	0(0)	0(0)	0(0)	0(0)
Waterlines (n=13)	0(0)	0(0)	12(9)	0(0)	1(<1)	0(0)
Total Conventional (n=133)	2(1)	55(41)	69(52)	2(1)	5(4)	0(0)
Organic						
Litter (n=95)	6(5)	63(50)	18(14)	4(3)	3(2)	1(<1) ^a
Feed (n=27)	1(<1)	0(0)	22(17)	0(0)	4(3)	0(0)
Source water (n=1)	0(0)	0(0)	1(<1)	0(0)	0(0)	0(0)
Waterlines (n=3)	0(0)	0(0)	1(<1)	1(<1)	0(0)	1(<1) ^b
Total Organic (n=126)	7(6)	63(50)	42(33)	5(4)	7(6)	2(2)

^a Low discrimination *E. gallinarum/faecium*

^b Low discrimination *E. durans/hirae*

Table 3: Minimal inhibitory concentration (MIC) ranges, MIC50s and MIC90s for 17 antibiotics determined for *E. faecalis* and *E. faecium* recovered from conventional (CONV) and newly organic (ORG) poultry farms.

Antimicrobial	Farm Type	<i>E. faecalis</i>			<i>E. faecium</i>		
		MIC Range	MIC50	MIC90	MIC Range	MIC50	MIC90
Chloramphenicol	CONV	4--≥64	8	8	4--16	8	8
	ORG	4--16	8	8	≤2--8	8	8
Ciprofloxacin	CONV	0.5--4	1	2	0.25--≥8	4	4
	ORG	1--≥8	1	2	0.5--≥8	2	4
Daptomycin	CONV	≤0.5--4	1	1	≤0.5--4	2	4
	ORG	≤0.5--4	1	2	≤0.5--4	2	4
Erythromycin	CONV	≤0.5--≥16	≥16	≥16	≤0.5--≥16	1	8
	ORG	≤0.5--≥16	1	≥16	≤0.5--≥16	2	4
Flavomycin ^a	CONV	≤1--≥32	2	8	2--≥32	≥32	≥32
	ORG	≤1--≥32	≤1	2	2--≥32	≥32	≥32
Gentamicin	CONV	≤128--≥2048	≤128	≤128	≤128--≥2048	≤128	1024
	ORG	≤128	≤128	≤128	≤128	≤128	≤128
Kanamycin	CONV	≤128--≥2048	≤128	≥2048	≤128--≥2048	256	≥2048
	ORG	≤128--≥2048	≤128	≤128	≤128--≥2048	256	256
Lincomycin ^b	CONV	16--≥64	≥64	≥64	≤1--≥64	≥64	≥64
	ORG	16--≥64	≥64	≥64	≤1--≥64	16	≥64
Linezolid	CONV	≤0.5--2	1	2	1--4	2	2
	ORG	1--4	2	4	1--4	2	4
Nitrofurantoin	CONV	8--≥128	8	64	32--≥128	≥128	≥128
	ORG	8--≥128	16	64	16--≥128	64	≥128
Penicillin	CONV	2--≥32	4	8	≤0.5--≥32	16	≥32
	ORG	2--8	4	8	≤0.5--16	4	8

Table 3 (continued)

Antimicrobial	Farm Type	<i>E. faecalis</i>			<i>E. faecium</i>		
		MIC Range	MIC50	MIC90	MIC Range	MIC50	MIC90
Streptomycin	CONV	≤512-->2048	≤512	2048	≤512-->2048	≤512	≤512
	ORG	≤512-->2048	≤512	1024	≤512-->2048	≤512	≤512
Quinupristin/ Dalfopristin ^b	CONV	2--32	4	16	≤1—32	4	8
	ORG	2--8	8	8	≤1—16	2	4
Tetracycline	CONV	≤4--≥64	≥64	≥64	≤4--≥64	≥64	≥64
	ORG	32--≥64	≥64	≥64	≤4--≥64	≤4	32
Tigecycline	CONV	0.03—0.5	0.12	0.25	0.06—0.5	0.12	0.25
	ORG	0.06—0.25	0.25	0.25	0.03—0.25	0.12	0.25
Tylosin	CONV	1--≥64	≥64	≥64	1--≥64	4	≥64
	ORG	1--≥64	4	≥64	2—16	4	8
Vancomycin	CONV	≤0.5—4	1	2	≤0.5—2	≤0.5	1
	ORG	1—4	1	2	≤0.5—4	1	2

^a *E. faecium* are intrinsically resistant to flavomycin.

^b *E. faecalis* are intrinsically resistant to lincomycin and quinupristin/dalfopristin.

Table 4: Antibiotic-resistant *E. faecalis* and *E. faecium* isolated from different environmental sample types recovered from conventional (CONV) and newly organic (ORG) poultry farms.^a

Antimicrobial	Farm Type	Number (%) of resistant <i>E. faecalis</i>				Number (%) of resistant <i>E. faecium</i>			
		Litter	Feed	Source water	Waterlines	Litter	Feed	Source water	Waterlines
Chloramphenicol	CONV	0(0)	2(20)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	ORG	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Ciprofloxacin	CONV	3(7)	0(0)	0(0)	0(0)	26(62)	8(53)	0(0)	5(42)
	ORG	3(5)	0(0)	0(0)	0(0)	9(41)	0(0)	0(0)	0(0)
Erythromycin	CONV	27(60)	10(100)	0(0)	0(0)	4(10)	4(27)	0(0)	1(0)
	ORG	11(17)	0(0)	0(0)	0(0)	1(6)	3(14)	0(0)	0(0)
Flavomycin ^a	CONV	4(9)	1(10)	0(0)	0(0)	21(50)	14(93)	0(0)	8(67)
	ORG	1(2)	0(0)	0(0)	0(0)	14(78)	22(100)	0(0)	0(0)
Gentamicin	CONV	5(11)	0(0)	0(0)	0(0)	9(21)	0(0)	0(0)	3(25)
	ORG	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Kanamycin	CONV	11(24)	1(10)	0(0)	0(0)	14(33)	1(7)	0(0)	3(25)
	ORG	5(8)	0(0)	0(0)	0(0)	0(0)	2(9)	0(0)	0(0)
Lincomycin ^b	CONV	45(100)	10(100)	0(0)	0(0)	40(95)	15(100)	0(0)	8(67)
	ORG	63(100)	0(0)	0(0)	0(0)	9(50)	22(100)	1(100)	0(0)
Nitrofurantoin	CONV	3(7)	2(20)	0(0)	0(0)	31(74)	7(47)	0(0)	8(67)
	ORG	3(5)	0(0)	0(0)	0(0)	7(39)	3(14)	0(0)	0(0)
Penicillin	CONV	3(7)	0(0)	0(0)	0(0)	22(52)	5(33)	0(0)	9(75)
	ORG	0(0)	0(0)	0(0)	0(0)	1(6)	0(0)	0(0)	0(0)

Table 4 (continued)

Antimicrobial	Farm Type	Number (%) of resistant <i>E. faecalis</i>				Number (%) of resistant <i>E. faecium</i>			
		Litter	Feed	Source water	Waterlines	Litter	Feed	Source water	Waterlines
Streptomycin	CONV	16(36)	1(10)	0(0)	0(0)	4(10)	1(7)	0(0)	1(8)
	ORG	7(11)	0(0)	0(0)	0(0)	1(6)	0(0)	0(0)	0(0)
Quinupristin/ Dalfopristin ^b	CONV	7(16)	10(100)	0(0)	0(0)	8(19)	5(33)	0(0)	1(8)
	ORG	38(60)	0(0)	0(0)	0(0)	1(6)	2(9)	0(0)	0(0)
Tetracycline	CONV	43(96)	10(100)	0(0)	0(0)	36(86)	11(73)	0(0)	9(75)
	ORG	63(100)	0(0)	0(0)	0(0)	4(22)	1(5)	0(0)	0(0)
Tylosin	CONV	29(64)	10(100)	0(0)	0(0)	3(7)	3(20)	0(0)	1(8)
	ORG	13(21)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)

^a *E. faecium* are intrinsically resistant to flavomycin.

^b *E. faecalis* are intrinsically resistant to lincomycin and quinupristin/dalfopristin.

Table 5: Predominant (>2 isolates) acquired multi-drug resistance (MDR) patterns among *E. faecalis* and *E. faecium* isolated from conventional (CONV) and newly organic (ORG) poultry farms.^a

Farm Type	Species	MDR Pattern	Number (% of species from farm type)
CONV	<i>E. faecalis</i>	ERY-GEN-KAN-STR-TET-TYL	3(5)
		ERY-KAN-STR-TET-TYL	4(7)
ORG	<i>E. faecalis</i>	ERY-KAN-STR-TET-TYL	5(8)
CONV	<i>E. faecium</i>	CIP-GEN-KAN-LIN-TET-NIT	4(6)
		CIP-LIN-PEN-TET-NIT	10(14)
		CIP-LIN-TET-NIT	5(7)
		LIN-PEN-TET-NIT	3(4)
		LIN-SYN-TET-NIT	5(7)
ORG	<i>E. faecium</i>	CIP-LIN-NIT	3(7)

^a *E. faecalis* are intrinsically resistant to lincomycin (LYN) and streptogramin A (dalfopristin) (SYN) (Dina et al. 2003), and *E. faecium* are intrinsically resistant to flavomycin (FLA); therefore, these species/drug combinations were excluded from the MDR analysis.

Figure Legends:

Figure 1: Percentage of *E. faecalis* (Panel A) and *E. faecium* (Panel B) from conventional and newly organic poultry houses expressing acquired resistance to a particular antibiotic (* denotes p -value <0.05 ; ** denotes p -value <0.01 ; *** denotes p -value <0.001). *E. faecalis* is intrinsically resistant to lincomycin and streptogramin A (dalfopristin) (Dina et al. 2003). *E. faecium* is intrinsically resistant to flavomycin.

Figure 2: Percentage of multi-drug resistant (to 0 3 antimicrobial classes) *E. faecalis* and *E. faecium* recovered from conventional and newly organic poultry houses (* denotes p -value $=0.02$, ** denotes p -value <0.001).

Figure 3: Percentage of *E. faecalis* (Panel A) and *E. faecium* (Panel B) from conventional and newly organic poultry houses expressing acquired resistance to varying numbers of antibiotics.

Figure 1

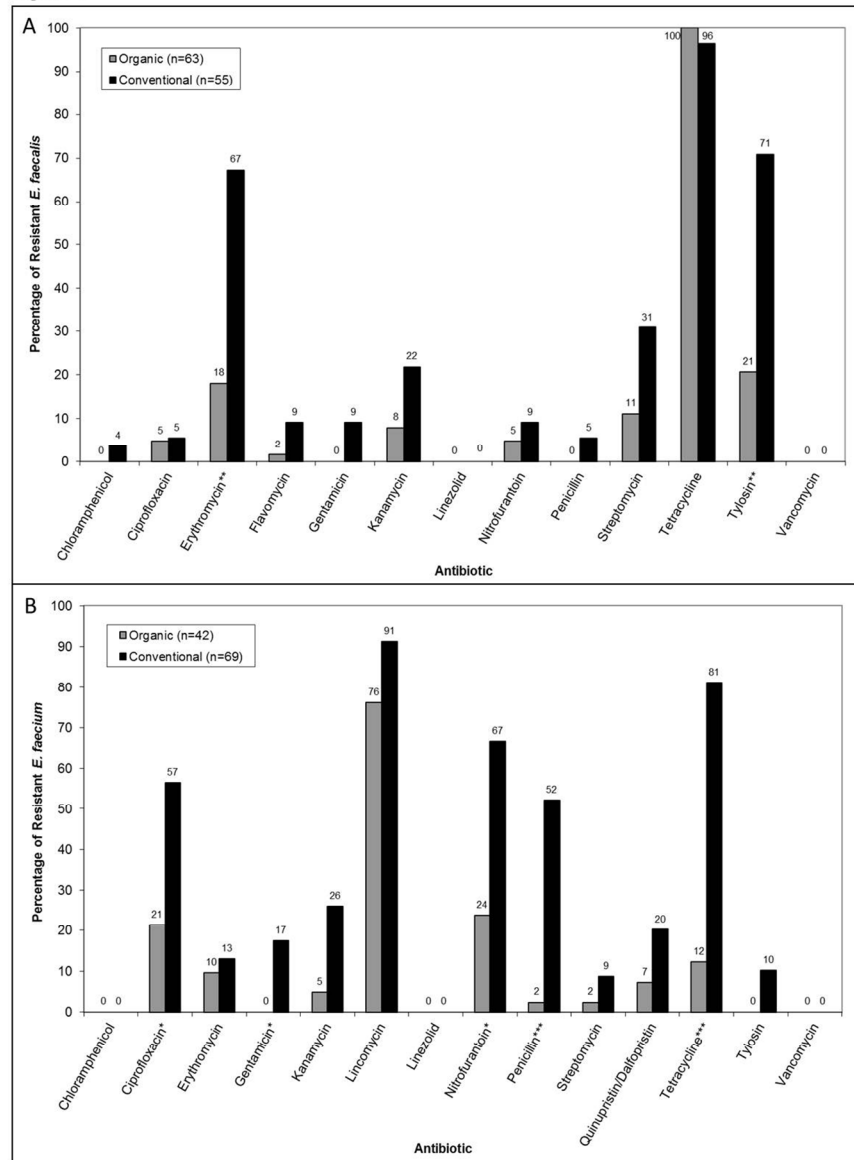


Figure 1: Percentage of *E. faecalis* (Panel A) and *E. faecium* (Panel B) from conventional and newly organic poultry houses expressing acquired resistance to a particular antibiotic (* denotes p-value <0.05; ** denotes p-value <0.01; *** denotes p-value <0.001). *E. faecalis* is intrinsically resistant to lincomycin and streptogramin A (dalfopristin) (Dina et al. 2003). *E. faecium* is intrinsically resistant to flavomycin.
200x277mm (150 x 150 DPI)

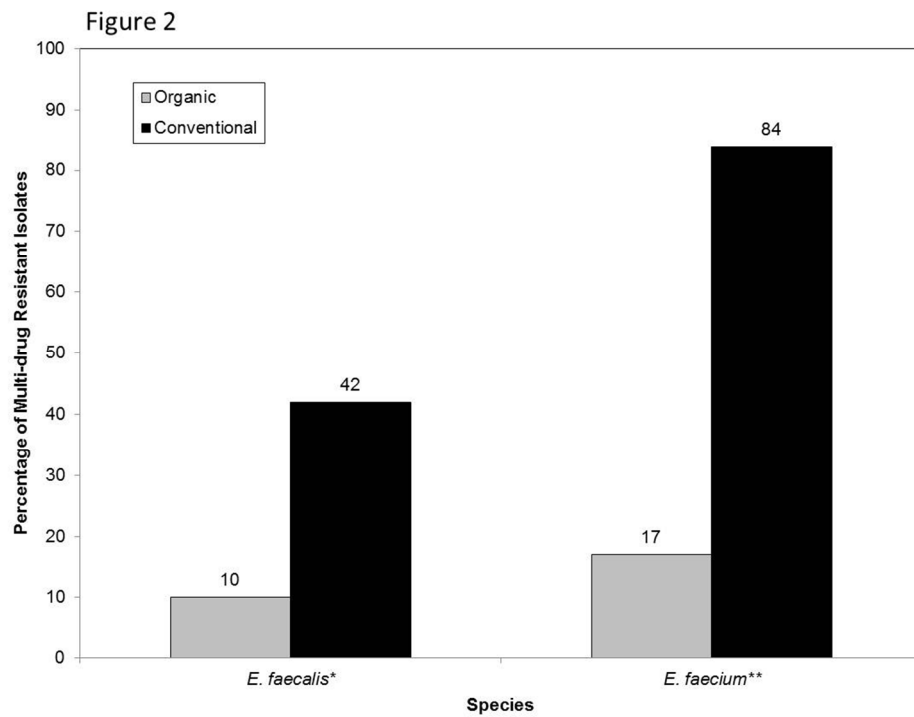


Figure 2: Percentage of multi-drug resistant (to 3 antimicrobial classes) *E. faecalis* and *E. faecium* recovered from conventional and newly organic poultry houses (* denotes p-value =0.02, ** denotes p-value <0.001).
199x154mm (150 x 150 DPI)

Figure 3

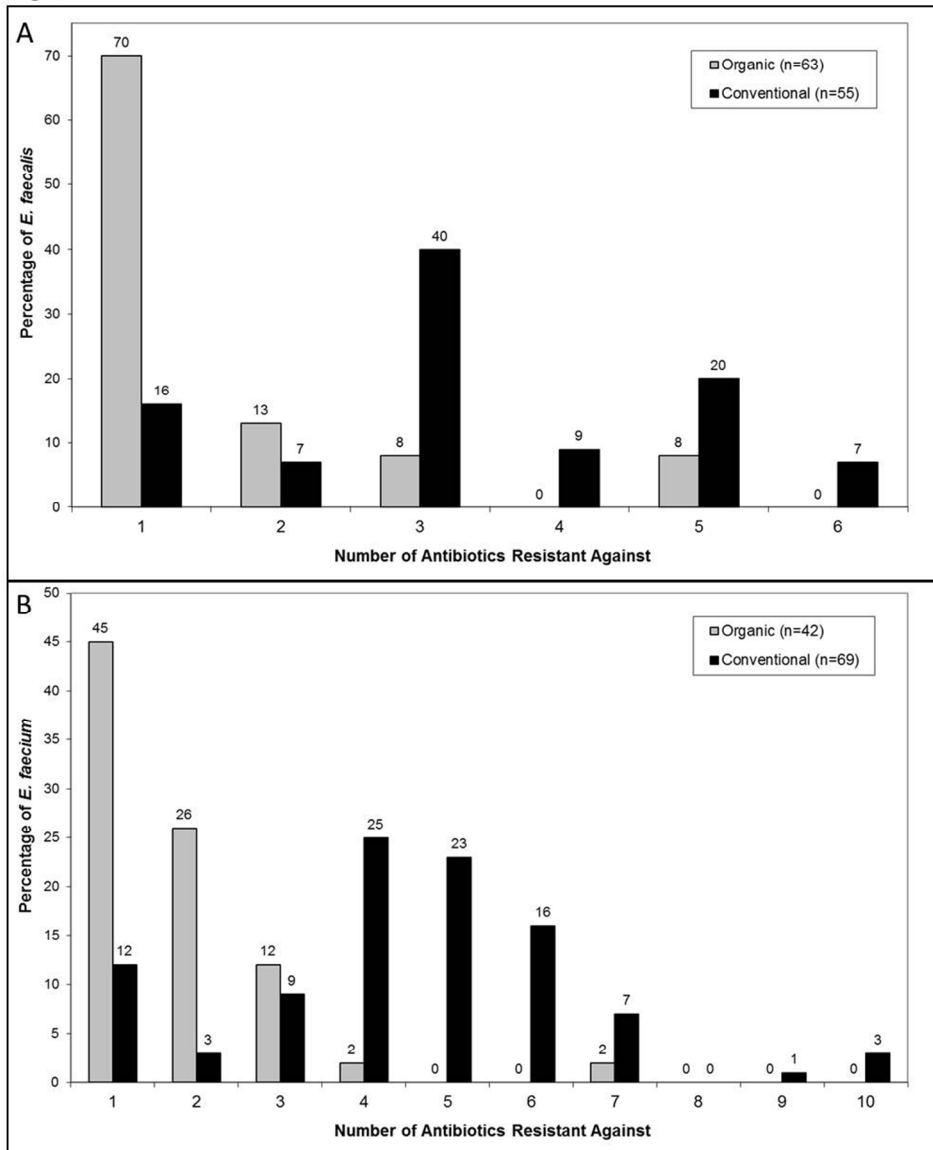


Figure 3: Percentage of *E. faecalis* (Panel A) and *E. faecium* (Panel B) from conventional and newly organic poultry houses expressing acquired resistance to varying numbers of antibiotics.
172x215mm (150 x 150 DPI)