

12-2016

Prevalence and antimicrobial resistance patterns of *Salmonella* isolated from poultry farms

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**PURDUE UNIVERSITY
GRADUATE SCHOOL
Thesis/Dissertation Acceptance**

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By CARMEN G VELASQUEZ MORENO

Entitled

PREVALENCE AND ANTIMICROBIAL RESISTANCE PATTERNS OF SALMONELLA ISOLATED FROM POULTRY FARMS

For the degree of Master of Science

Is approved by the final examining committee:

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11/28/2016

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PREVALENCE AND ANTIMICROBIAL RESISTANCE PATTERNS OF *SALMONELLA* ISOLATED
FROM POULTRY FARMS

A Thesis

Submitted to the Faculty

of

Purdue University

by

Carmen G Velasquez Moreno

In Partial Fulfillment of the

Requirements for the Degree

of

Master of Science

December 2016

Purdue University

West Lafayette, Indiana

Para mis padres, mis hermanos y mi mejor amiga; mi hermana. Este es nuestro logro, gracias a su apoyo y amor incondicional. Para Dios por todas y cada una de sus bendiciones a lo largo de este proceso.

ACKNOWLEDGEMENTS

This thesis is the result of Dr. Manpreet Singh's trust in me and my capabilities. Thank you Dr. Singh for your support and guidance and for giving me this opportunity. I want to thank my committee members; Dr. Haley Oliver, Dr. Fernanda San-Martín, and Dr. Paul Ebner for their help and suggestions. This work couldn't be done without the help of the most awesome people on earth; my lab team. Thank you for all your constant help, advice, and more importantly, for your friendship. I am beyond grateful for Rhonda Taylor, for all her support inside and outside the lab and for Matthew Bailey for all his mentoring through all this process. Thank you Jagpinder Brar and Sydney Corkran for all your help with this study. Thank you Estefania Novoa for all your time and help and Morgan Redemann for your friendship. I will walk five hundred miles for all of you guys.

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LIST OF ABBREVIATIONS

AMP	Ampicillin
AMR	Antimicrobial Resistance
AUG	Amoxicillin/clavulanic acid
AXO	Ceftriaxone
AZI	Azithromycin
CDC	Center for Disease Control and Prevention
CHL	Chloramphenicol
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
FIS	Sulfisoxazole
FOX	Cefoxitin
GEN	Gentamicin
MDR	Multidrug Resistance
MIC	Minimum Inhibitory Concentration
NAL	Nalidixic Acid
NARMS	National Antimicrobial Resistance Monitoring System
NVSL	National Veterinary Services Laboratory

PCR Polymerase Chain Reaction

STR Streptomycin

SXT Trimethoprim/sulfamethoxazole

TET Tetracycline

USDA United States Department of Agriculture

WHO World Health Organization

XNL Ceftiofur

ABSTRACT

Velasquez Moreno, Carmen G. M.S., Purdue University, December 2016. Prevalence and Antimicrobial Resistance Patterns of *Salmonella* Isolated from Poultry Farms. Major Professor: Manpreet Singh.

Antimicrobial agents are used in human medicine to treat infections by inhibiting the growth of bacteria. The same or similar drugs are used in in food-producing animals for therapeutic and sub-therapeutic purposes. Antimicrobial resistant bacteria that develop on farms are a public health risk as these organisms may contribute to treatment failures in infected humans. The main objective of this study was to determine the prevalence of *Salmonella* isolated from poultry farms in two different seasons to evaluate the effect of recommended biosecurity practices. Specifically, we identified *Salmonella* serotypes, measured antimicrobial resistance (AMR), and characterized AMR patterns. We hypothesized that implementing and improving biosecurity practices on poultry farms decreases *Salmonella* prevalence, resulting in a concurrent decrease in AMR *Salmonella* populations. Cloacal swabs, drag swabs, and litter samples were obtained from four different farms from the same integrator. These farms were sampled on three different days during one grow-out period during March-April (pre-recommendations season).

After sampling, recommendations for improvement of the biosecurity practices were made and a second sampling was performed during October-November (post-recommendations season). Recommendations included, but were not limited to improvement of insect and rodent control, restrictions on farms visitors, restrictions on contact with poultry, and logging of entry and exit to farms.

Presumptive *Salmonella* isolates obtained on selective agars (XLT4) were confirmed by performing PCR targeting the *hliA* gene. After confirmation, all positive *Salmonella* were serotyped and antimicrobial resistance was performed using the Sensititre™ micro-dilution system and NARMS plates. Higher *Salmonella* prevalence values were observed for the post-recommendations season, however, no significant differences ($p>0.05$) were observed between seasons. Overall, six different *Salmonella* serotypes were identified. *S. Enteritidis* was the most common *Salmonella* serotype followed by *S. Berta* and *S. Mbandaka*. These three *Salmonella* serotypes were present on all four farms. *S. Typhimurium*, *S. Kentucky*, and *S. Tennessee* were each found on a single farm. A total of 7% isolates exhibited antimicrobial resistance with a single isolate showing multidrug resistance. Resistance values were highest for tetracycline, nalidixic acid, and streptomycin.

CHAPTER 1. INTRODUCTION

According to Scallan et al. (2011), of all foodborne illnesses in the US, Salmonellosis causes the highest number of hospitalizations. *Salmonella* outbreaks are often associated with the consumption of contaminated poultry products. At the same time, antimicrobial resistance (AMR) pathogens and their treatment is of growing concern. Antimicrobial resistance is the ability of bacteria to resist the bacteriocidal or bacteriostatic effects of antimicrobials. AMR *Salmonella* is a public health risk, especially with respect to antimicrobials commonly used to treat salmonellosis such as ciprofloxacin, ceftriaxone, nalidixic acid, azithromycin, penicillin, and trimethoprim/sulfamethoxazole (FDA, 2013).

The main objective of this study was to determine the changes in *Salmonella* prevalence in poultry farms following recommendations for improving biosecurity and management practices. *Salmonella* prevalence, both pre- and post-recommendations, was determined via:

- 1) Confirmation of *Salmonella* by molecular techniques;
- 2) Identification of *Salmonella* serotypes and their association with sample type; and
- 3) Distinguishing patterns of antimicrobial resistance in *Salmonella*.

CHAPTER 2. LITERATURE REVIEW

2.1 Salmonella

In 1885, Daniel E. Salmon, an American scientist, isolated a pathogen from the intestine of a pig. In 1900, Joseph Léon Marcel Lignières, a French bacteriologist, suggested that the swine cholera organism should be named "*Salmonella*" to honor Daniel Salmon (White, 1926). While originally associated with pigs, *Salmonella* has been isolated from soil, water, and sewage mainly because of contamination with fecal matter (Li et al., 2013). *Salmonella* belongs to the *Enterobacteriaceae* family and is a Gram-negative, motile, facultative anaerobe, with peritrichous flagella. Optimal temperature for its growth is 37°C. *Salmonella* can be differentiated from other bacteria based on biochemical properties such as hydrogen sulfide production, catalase production and lack of oxidase production (Li et al., 2013).

Salmonellosis is the result of *Salmonella* infection. Once introduced into the host, *Salmonella* usually undergoes an incubation time varying from 8 to 72 h. Clinical symptoms may include nausea, abdominal pain, and watery diarrhea. This infection is more severe in immunocompromised, young, or elderly people (D'Aoust, 1991).

Salmonella can be present in raw food, mostly meats and fresh produce that become contaminated through human or animal feces. With the aim of minimizing *Salmonella* loads in raw food, it is important to maintain animal husbandry and good agricultural practices (Bell & Kyriakides, 2002).

Based on estimates, *Salmonella* is the leading cause of bacterial foodborne illness worldwide with around 80 million cases, of which 155,000 results in deaths. Worldwide, *Salmonella* is responsible of approximately 3% of foodborne infections. This foodborne pathogen is regularly found in and transmitted through foods in both developed and developing countries (Majowicz et al. 2010). In the United States, non-typhoidal *Salmonella* results in a million illnesses each year. *Salmonella* is the leading cause of deaths linked to foodborne illnesses (Scallan et al., 2011) with 19,000 hospitalizations and 378 deaths. Non-typhoidal *Salmonella* also poses a significant economic burden with a total of \$3.7 billion per year (Hoffman et al., 2015). Non-typhoidal *Salmonella* poses the largest disease burden, suggesting that the pathogen is ubiquitously present and the severity of its illness (WHO, 2015b).

2.2 Salmonella in the poultry industry

According to the United States Department of Agriculture (USDA), United States is the largest producer of poultry producing over 43 billion pounds. Production is concentrated primarily in the Southeast US, specifically in Georgia, Arkansas, Alabama, Mississippi, and North Carolina (USDA, 2015).

Host specific infections in chickens can occur due to *S. Pullorum* and *S. Gallinarum*, however, infections with other less species-specific serovars such as *S. Typhimurium*, *S. Enteritidis*, and *S. Heidelberg*, are also common (Foley et al., 2008; CDC, 2011). In 2007 A wide range of serotypes were found in poultry in the US, with *S. Enteritidis*, *S. Newport*, *S. Heidelberg*, and *S. Typhimurium* being associated with clinical isolates (CDC, 2011). In 1960 *S. Enteritidis* was a very rare serovar among the poultry industry, but since the eradication of *S. Pullorum* and *S. Gallinarum* in 1990, *S. Enteritidis* has become the most common serovar isolated from poultry (Foley et al., 2008). *Salmonella* infection in poultry is most common in one-day old chickens where the infection goes from the caeca to the internal tissues. However, 3-4 days old animals can become carriers without being infected due to the high level of maturity of the cells (Shivaprasada et al., 2013). Some factors that contribute to the development of salmonellosis include age and dose, with older birds less prone to developing salmonellosis even with concentrations of 10^6 *S. Typhimurium* (Sadler et al., 1969).

The same correlation was found by Barrow (1992) based on ELISA analysis between circulating antibody and recovery of the tissue. In addition to the age and dose, the *Salmonella* serovar and strain are main factors that determine the development of *Salmonella* infection. Colonization can be enhanced by less invasive serovars, compared to more invasive serovars (Barrow et al., 1988).

According to submissions to the National Veterinary Service Laboratories (NVSL), from January 1 to December 31, 2009, 4761 isolates from chicken were submitted for serotyping test, of which 154 were non-clinical isolates, and 4607 were identified as clinical isolates (Erdman et al., 2009). From the non-clinical cases, the most common serovars found in chicken in 2009 in the US were *S. Enteritidis*, *S. Kentucky*, *S. Heidelberg*, *S. Senftenberg*, *S. Mbandaka*, *S. Montevideo*, *S. Schwarzengrund*, *S. Typhimurium*, *S. Anatum*, and *S. Berta*. In terms of clinical isolates, the most common serovars were *S. Enteritidis*, *S. Heidelberg*, *S. Kentucky*, *S. Senftenberg*, and *S. Typhimurium* (Erdman et al., 2009). Furthermore, a report by the CDC in 2011 showed *S. Enteritidis*, *S. Typhimurium*, *S. Newport*, and *S. Javiana* as the most common serovars isolated in chicken from clinical animal infections according to USDA-APHIS. For *Salmonella* isolated from clinical non-human sources in chicken, the most prevalent serovars were, *S. Enteritidis*, *S. Newport*, *S. Javiana*, and *S. Typhimurium* (CDC, 2013e).

Poultry is a significant cause of human infections with *Salmonella*. A multistate outbreak involving 30 states was linked to live poultry in 2013 leading to 158 cases and 29 hospitalizations (CDC, 2013c). A total of 95% of the people involved in the outbreak, bought chicks and duckling from a hatchery in Ohio that were infected with *S. Infantis*, *S. Lille*, *S. Newport* and *S. Mbandaka*, and were the strains involved in the outbreak (CDC, 2013c). A different multistate outbreak was also linked to live poultry occurring in the same year, resulting in 356 illnesses and 62 hospitalizations in 39 states. In this instance, a single strain of *S. Typhimurium* was identified as the origin of this outbreak, where 95% of the cases reported buying live poultry from agricultural feed stores (CDC, 2013d). *Salmonella* is highly associated with the poultry industry and efforts to control prevalence of this pathogen are focused on poultry processing. However, these actions to decrease *Salmonella* during processing is difficult to achieve, therefore eliminating *Salmonella* has been replaced by reduction of cross-contamination. In order to reduce *Salmonella* loads during processing it is important to maintain *Salmonella*-free chickens during pre-harvest. Therefore, biosecurity practices in different poultry operations such as feed, rodent and insect control and transportation have a direct effect of reducing *Salmonella* (Bailey et al., 1993).

2.3 Antimicrobial resistance

An antimicrobial is a low molecular weight substance, which can either inhibit growth or kill bacteria (Schwarz & Chaslus-Dancla, 2001). Antimicrobials include antibacterial drugs, antiviral agents, antifungal agents, and anti-parasitic drugs (CDC, 2013b). According to the US Food and Drug Administration (USFDA), antimicrobials are substances that have a wider spectrum of action among microorganisms such as bacteria, viruses, fungi, and parasites. On the other hand, antibiotics have a limited spectrum, acting mainly against bacteria. All antibiotics are antimicrobials, but not all antimicrobials are antibiotics (FDA, 2015).

Antimicrobial resistance (AMR) is the ability of the bacterial cell to withstand the bacteriocidal or bacteriostatic effect of antimicrobial drugs (FDA, 2015; CDC, 2015a; WHO, 2015a). Antimicrobial resistance is an increasing problem related to both, human and veterinary medicine (Silbergeld et al., 2008). Multi-drug resistance (MDR), is the ability of bacteria to simultaneously withstand effects of several antimicrobials (CDC, 2010).

There are many ways to classify antimicrobials with many common classification schemes based on mode of action (Collignon et al., 2009). Accordingly, there are four different modes of action (Tenover, 2006):

- Interference with cell wall synthesis: beta-lactams (penicillins, cephalosporins, carbapenems, and monobactams) interfere with peptidoglycan layer synthesis by limiting enzymes action.
- Inhibition of protein synthesis: macrolides, aminoglycosides, tetracyclines, chloramphenicol, streptogramins and oxazolidinones inhibit protein synthesis using their antimicrobial effect by interfering with acid nucleic synthesis. Macrolides, aminoglycosides and tetracyclines work in similar way by binding to the ribosome in the 30S subunit, but different to chloramphenicol which binds to 50S subunit.
- Interference with nucleic acid synthesis: fluoroquinolones make use of their antimicrobial effect to disturb DNA synthesis.
- Inhibition of a metabolic pathway: Sulfonamides and trimethoprim block the metabolic pathway for folic acid synthesis in order to block DNA synthesis. The combination of trimethoprim and sulfamethoxazole inhibits this same pathway in two steps.

For non-typhoidal *Salmonella*, resistance to ampicillin, chloramphenicol, streptomycin, sulfonamide, and tetracycline represents an important MDR phenotype showing resistance to at least 5 classes of antimicrobials that are clinically important according to the classification in the Clinical and Laboratory Standards Institute (CLSI) (CDC, 2010). Another important MDR phenotype is resistance to ampicillin, chloramphenicol, streptomycin, sulfonamide, tetracycline, amoxicillin-clavulanic acid, and ceftriaxone (CDC, 2010). When MDR bacteria are implicated in infections or outbreaks, the alternatives of treatment become limited (Grace, 2013).

Antimicrobial resistance can occur due to mutations in bacteria, acquiring genetic information that encodes resistance from other bacteria (Tenover, 2006), and modification of target structures (Schwarz & Chalus-Dancla, 2001). This mechanism results from the selective pressure generated by the use of antimicrobials (Michael et al., 2013). Resistance genes can originate from bacteria that produce the antimicrobial since they carry resistance genes to protect themselves (Schwarz et al., 2001). Resistance genes code for proteins that mediate resistance to an antimicrobial agent or class. These resistance genes can spread to other bacteria by acquiring them through mobile genetic elements (Schwarz et al., 2001). Mobile genetic elements such as plasmids and transposons have been detected from bacteria from the pre-antimicrobial area (Schwarz et al., 2001).

Mutation or stepwise mutation is a second way to gain AMR, which helps the bacteria to adapt to the host. The genes responsible for physiological cell metabolism are modified in order to change the products from the substrate metabolites to specific antimicrobials (Schwarz & Chaslus-Dancla, 2001). Similarly, bacteria can develop resistance to synthetic antimicrobials such as fluoroquinolones by complex mutations of the target gene in the chromosome that results in resistant DNA topoisomerases to this antimicrobial class (Schwarz et al., 2001). A third mechanism to develop resistance is modifying the target structures by single or multistep mutations, which allow the bacteria to gain resistance to that specific antimicrobial agent or class (Schwarz & Chaslus-Duncla, 2001). Other mechanisms consist of preventing the antimicrobial to reach the target site and avoid its effect, either by expelling the antimicrobial agent from the cell or by mutations (Tenover, 2006). A strain resistant to an antimicrobial agent is predisposed to present resistance to other antimicrobial agents of that same antimicrobial class (Tenover, 2006).

Multidrug resistant bacteria can be the result of two main mechanisms. The first mechanism is the accumulation of multiple genes in which each gene codes for resistance to a specific drug, taking place in resistance plasmids or transposons. A second mechanism can occur as a result of the increased expression of genes coding for multidrug efflux pumps, being able to pump more than one type of antimicrobial (Nikaido, 2009).

2.3.1 Resistance to tetracyclines

Tetracyclines are a broad-spectrum antimicrobial class that includes tetracycline, minocycline, and other substances (CDC, 2013b). For efflux proteins in Gram-negative bacteria, 8 *tet* genes have been sequenced, these being *tet* (A-E, G, H) and *tet* (J). Each of these 8 genes has a *tet* repressor gene. When tetracycline is not present, the prescription of the *tet* structural gene is blocked by the *tet* repressor protein, which is bound to the tetracycline Mg^{2+} , the base of the tetracycline inducible expression (Roberts, 1996). Furthermore, in ribosome protection, 8 different classes of proteins have been known such as M, O, P, Q, S, T, W, and *otrA* (Levy et al., 1999).

2.3.2 Resistance to macrolides

Macrolides are antimicrobials that are regularly used as a treatment for Gram-positive bacteria and other infections related to the respiratory tract. These antimicrobials are used as a substitute in people allergic to penicillin (CDC, 2013b). Resistance to macrolides is based on three main mechanisms such as target modification, active efflux, and enzymatic inactivation (Roberts et al., 1999). In Gram-positive and negative bacteria, target modification by rRNA methylases has been observed. Ten different active efflux mechanisms confer resistance to macrolides differing in the substrate spectra. Genes of transporter proteins are found in plasmids. Ultimately, enzymatic inactivation occurs by action of different enzymes such as esterases, hydrolases, and tranferases, each showing a specific substrate spectrum.

For esterase enzymes, the genes *ere(A)* and *ere(B)* are present in *Enterobacteriaceae* (Schwarz & Chaslus-Dancla, 2001).

2.3.3 Resistance to penicillins

Resistance to penicillins, is the result of inhibition of wall synthesis in bacterial cells. This inhibition occurs through interferences with enzymes responsible for peptidoglycan layer synthesis (Tenover, 2006). Betalactam antibiotics bind to transpeptidase, which is the main penicillin-binding protein used to catalyze the link of sugar and peptide present in the peptidoglycan molecule. Finally, this binding interferes with peptidoglycan, therefore interfering with wall synthesis (McManus, 1997).

2.3.4 Resistance to aminoglycosides

Aminoglycosides are substances used against Gram-negative bacteria (CDC, 2013b). Resistance to these antimicrobials is due to the inhibition of protein synthesis. These antimicrobials bind to the 30S subunit in the ribosome (Tenover, 2006). In order for aminoglycosides to reach the ribosome and inhibit cell-wall synthesis, these antimicrobials have to cross the cytoplasmic membrane (McManus, 1997).

2.3.5 Resistance to sulfonamides and trimethoprim

Sulfonamides inhibit dihydropteroic acid synthetase (DHPS) whereas trimethoprim inhibits the dihydrofolate reductase (DHFR) (Schwarz & Chaslus-Dancla, 2001). The mechanism of resistance to these substances relies on chromosomal mutations, as well as plasmid-encoded DHPS for sulfonamides or DHFR for trimethoprim

(Huovinen et al., 1995). Mutations in *dfp* gene are associated with resistance to trimethoprim in different pathogens. Sulfonamides inhibit the formation of folic acid by restricting DHPS enzyme; resistance to this agent is due to chromosomal mutations in the gene *folP* for DHPS, which causes a decreased affinity between the expressed enzyme and the antimicrobial (Sköld, 2001).

2.3.6 Resistance to fluoroquinolones

Fluoroquinolones are a broad-spectrum antimicrobial used to treat severe infections, specifically infections acquired in hospitals (CDC, 2013b).

Since fluoroquinolones are synthetic substances, resistance to them is mainly due to mutations in primary and secondary target enzymes (Hooper, 2000). The complexity of the AMR mechanisms developed by pathogens is directly related to the increase in antimicrobials usage (Krause, 1992).

The spread of AMR can be accelerated by the location on mobile genetic elements such as plasmids, transposons, and integrons that contain one or more resistance genes. According to Schwartz et. al (2001), transfer of AMR bacteria between different hosts can occur either by contact with bacteria or contaminated surfaces such as skin or by ingestion of contaminated food with this bacteria.

2.4 Antimicrobial use in poultry

Antimicrobials have been utilized in human and veterinary medicine for therapeutic and prophylaxis of infectious diseases (van den Bogaard & Stobberingh, 2000). *Salmonella* infections in humans can be produced through direct contact with infected animals or contaminated animal feces, but most frequently due to the consumption of food products of animal origin (van den Bogaard & Stobberingh, 2000). The antimicrobials administered to animals for different reasons belong to the same class of antimicrobials that are being used to treat human infections (Schwarz et al., 2001). The use of antimicrobials for therapy and prophylaxis are usually given to animals through water or feed and with concentrations higher than those for growth promotion, which are administered through feed additives (Wegener, 2003).

Therapy, metaphylaxis, prophylaxis, and growth promotion are the four main reasons to use antimicrobials in animals (Schwarz et al., 2001). For therapeutic purposes, antimicrobials are given to individual or a group of animals with the objective of treat an infection. When antimicrobials are being administrated to a group of animals where only a few are showing symptoms of the disease, but a spreading of the disease is highly probable affecting the majority of the group, it is called metaphylaxis. With metaphylaxis, the number of infected animals is significantly reduced and thus the treatment costs. Prophylactic purposes include the use of antimicrobials as a preventive method; this can be administered for an individual or a group of animals. Furthermore,

for growth promotion purposes, antimicrobials are used as growth promoters only for food-producing animals (Schwarz et al., 2001). Guidelines regarding the use of antimicrobials are established, defining the type of animal, duration, and the dose states the maximum of an antimicrobial that can be administrated. Regarding the type and age of the animal, these guidelines contain a list of antimicrobial agents that are accepted and banned in food-producing animals (Schwarz et al., 2001). Use of extralabel antimicrobials is conditioned to veterinary supervision, antimicrobials also cannot be administrated in the feed, and residues that can result in a risk to public health can not be present. The FDA can prohibit the use of antimicrobial agents used in food-producing animals if the use of that specific agent results in a risk to public health because of AMR.

According to the FDA the most consumed antimicrobials in food producing animals are tetracyclines, followed by ionophores and penicillins (FDA,2014a). Use of fluoroquinolones in poultry is illegal, on the other hand, use of ceftiofur, an antimicrobial agent from the cephalosporins class, is approved in the poultry industry only in day-old chicks to control early mortality by *Escherichia coli* infections (FDA, 2014b).

2.5 Antimicrobial resistant *Salmonella*

Salmonella Enteritidis is the most common serovar among the poultry industry, specifically chicken (Ray, 2004). Because of the high frequency of use of antimicrobials

in birds, AMR *Salmonella* has increased, leading to MDR strains that have been confirmed to be resistant to chloramphenicol, tetracyclines, streptomycin, sulfonamides, and ampicillin (Ray, 2004). One of the most important and recent outbreaks regarding poultry products and *Salmonella* is the multistate outbreak of MDR *Salmonella* Heidelberg linked with chicken from Foster Farms. Covering 29 states, 634 persons were infected with 7 different strains of *Salmonella* Heidelberg leading to 38% of infected persons to be hospitalized, and 15% of the ill persons developed blood infections (CDC, 2014a). The National Antimicrobial Resistance Monitoring System (NARMS) laboratory from the Centers for Disease Control and Prevention (CDC) analyzed 68 isolates collected from humans infected with the seven different strains of *S. Heidelberg* and reported that 44 (65%) isolates exhibited AMR and 24 (35%) were MDR showing different patterns of resistance. Ampicillin, chloramphenicol, gentamicin, kanamycin, streptomycin, sulfisoxazole, and tetracycline were the antimicrobials that these isolates manifested resistance. Furthermore, NARMS received 5 isolates from 4 chicken products from people infected as a result of the Foster Farms chickens obtained from a store in California. Out of these 5 isolates, 4 (80%) exhibited AMR, with 1 (20%) being MDR. The 4 isolates showed different combinations of resistance to kanamycin, streptomycin, sulfisoxazole, and tetracycline. The same analysis was conducted for 8 out of 9 isolates collected from 9 retail samples of Foster Farms chicken in California. All the isolates (100%) were resistant to antimicrobials, with 4 (50%) being MDR. Similar to previous instances, different patterns of resistance were identified; resistance to ampicillin, chloramphenicol, gentamicin, kanamycin, streptomycin, sulfisoxazole, and

tetracycline was detected (CDC, 2014a). In 2014, an outbreak in Tennessee, led to 9 illnesses, with 22% hospitalizations due to an outbreak caused by *Salmonella* Heidelberg in mechanically separated chicken. After this outbreak, the company recalled 33,840 pounds of chicken and the NARMS laboratory analyzed these strains of *Salmonella* Heidelberg from all the ill people for AMR. From the 9 isolates collected, 2 (~20%) were MDR, and the other 7 (78%) were susceptible to all the antimicrobials tested (CDC, 2014b).

Fluoroquinolones and third generation cephalosporins are used in the US to treat severe *Salmonella* infections. According to NARMS reports (2013) from human isolates, 3% (61/2178) of *Salmonella* was nalidixic acid resistant where 36% (22/61) were *S. Enteritidis*. Furthermore, 3% (65/2236) of this *Salmonella* were resistant to ceftriaxone. With regards to MDR of non-typhoidal *Salmonella*, 10% isolates showed resistance to three or more antimicrobial classes (100). Antimicrobial resistant *Salmonella* has caused 1,200,000 infections each year, of which 100,000 infections are due to AMR non-typhoidal *Salmonella*, resulting in \$365 millions in medical costs. In order to treat *Salmonella* infections, ceftriaxone and ciprofloxacin are some of the main antimicrobial agents used in human medicine. Non-typhoidal *Salmonella* exhibits resistance to multiple antimicrobial agents, mainly ceftriaxone and ciprofloxacin. Around 5% of *Salmonella* tested by the CDC has been shown to have resistance to more than 5 types of antimicrobial agents (CDC, 2015a).

2.6 Impact of antimicrobial resistance

Infections with AMR bacteria lead to mortality rates and time of hospitalizations twice as great in comparison with infections not related to AMR (French, 2005). In order to control this public threat, antimicrobials agents should be used in a responsible manner, controlling the use of these agents in medical and nursing facilities (French, 2010). According to the CDC, more than 2,049,442 illnesses and 23,000 deaths are the results of AMR. Infections with AMR bacteria result in less alternatives to treat infections, as well as longer treatments and therefore a prolonged stay at hospitals, increasing the cost of therapy (CDC, 2015a).

Antimicrobial resistant bacteria can lead to an increased severity of diseases not related with gastrointestinal diseases, and thus an increase in illness burdens and outbreaks. Increase in AMR and treatment rates leads to the ineffectiveness of antimicrobial therapy (Hohmann, 2001), therefore awareness of antimicrobials important for clinical purposes such as fluoroquinolones, cephalosporins, and macrolides is important. Ineffectiveness of fluoroquinolones for salmonellosis treatment has been recorded since 1990. Resistance to fluoroquinolones coincides with isolates resistant to nalidixic acid that at the same time are susceptible to ciprofloxacin (Aaerstrup et al., 2003). Furthermore, Barza and Travers (2002), reported that using antimicrobials can increase susceptibility to foodborne illnesses, which can have either a competitive effect which is not linked to AMR or selective effect, meaning that AMR

pathogens will infect persons with antimicrobial therapy not only for infection treatment but any condition. Another important aspect of antimicrobial therapies is the narrow-spectrum versus broad-spectrum. Because antimicrobials interfere with intestinal flora, it is important to use antimicrobials with narrow-spectrum to decrease modification of the intestinal flora, and therefore avoid colonization of pathogenic bacteria (Mølbak, 2005). Misuse of antimicrobials in humans and AMR pathogens result in significant consequences, firstly, it contributes to the dissemination of AMR bacteria between individuals under treatments that are not because of foodborne illnesses which can face severe consequences for infections with these bacteria, and secondly an increase in outbreaks in clinical facilities where antimicrobials are used (Mølbak, 2005).

The European Union feed additives regulation established that antimicrobials used for infection treatments in humans or animals should not be used for growth promotion. Since antimicrobials intended to treat infections are being used for efficacy in production purposes, determining the impact on AMR bacteria in food-producing animals becomes a hard task (Wegener, 2003). A national public monitoring system for antimicrobial resistance was established in 1996. The NARMS - a collaboration between the FDA, CDC, and USDA, tracks AMR changes in bacteria from ill people, retail meats and food animals in the US. The FDA regulates the use of antimicrobials in humans and animals. Regulations for food-producing animals include the approved use of antimicrobials to treat sick animals, control a disease of a group of animals when some animals within that group are sick, prevent disease of a healthy group of animals that

are at risk of becoming ill, and promote growth or weight gain in a herd or flock of animals (CDC, 2016). Information provided by NARMS is of high importance for the FDA to create regulations based on the use of antimicrobials to maintain its effectiveness for humans and animals (FDA, 2016), as well as the WHO lists to reduce human health threat of AMR (Collignon et al., 2009). Since 2003, because of the impact on AMR by using antimicrobials in food-producing animals, the FDA has recommended that antimicrobials of importance in human health should be of limited use in food-producing animals when necessary to ensure animal health and veterinary assistance is involved (CDC, 2016). Antimicrobials that are being used in food-producing animals such as fluoroquinolones, macrolides, and cepheems are important for human health, and their use in animals need to be addressed most urgently. To treat *Salmonella* infections, antimicrobials are being administered to infected humans, specifically fluoroquinolones and some antimicrobial agents from cepheems class, yet these same antimicrobials are being administered to animals, leading to AMR. Critically important antimicrobial classes that are used in human health are aminoglycosides, ansamycins, carbapenems, cephalosporins, glycopeptides, lipopeptides, macrolides, oxazolidinones, penicillins, quinolones, streptogramins, and tetracyclines (Collignon et al., 2009)

The WHO classifies antimicrobials according to the importance of these to human health as critically important, highly important, and important. This classification was made for risk management guidelines and for use of antimicrobials in food-producing animals. With respect to prioritization, antimicrobials are critical under three main conditions; first, if an antimicrobial agent is the only or one of few alternatives to treat a large group of sick people, secondly, if the frequency of use of that antimicrobial is high, and lastly if an antimicrobial is used to treat an infection caused by a pathogen that can be transferred to humans from non-human sources, such as *Salmonella* (Collignon et al., 2009). According to this classification concerning the prioritization, quinolones, cephalosporins, and macrolides are the classes of which risk managements are extremely needed (Collignon et al., 2009). Limiting the use of antimicrobial for growth promotion purposes results in a decrease in the selective pressure, which leads to low the occurrence of AMR bacteria in food-producing animals (Emborg et al., 2001). The termination of antimicrobials for growth promotion in broilers in Denmark, showed no changes in mortality or weight gain, but it had an impact on feed efficacy. Reducing the use of antimicrobials in animals for no other purpose but therapeutic purposes results in lower levels of resistance in animals and therefore in humans (Emborg et al., 2001).

CHAPTER 3. PREVALENCE AND ANTIMICROBIAL RESISTANCE PATTERNS OF
SALMONELLA ISOLATED FROM POULTRY FARMS.

3.1 Abstract

Velasquez Moreno, Carmen G. M.S., Purdue University, December 2016. Prevalence and Antimicrobial Resistance Patterns of *Salmonella* Isolated from Poultry Farms. Major Professor: Manpreet Singh.

Approximately 100,000 human infections are attributed to antimicrobial resistant *Salmonella*, therefore, posing a severe risk to public health and decreasing the choices for treatment. The main objective of this study was to determine changes in *Salmonella* prevalence in poultry farms following recommendations to changes in biosecurity practices. Additionally, *Salmonella* serotypes were identified and antimicrobial resistance (AMR) patterns were described. We hypothesized that implementing and improving biosecurity measures and management in poultry farms decreases *Salmonella* prevalence.

Four different poultry farms in Southeast US from the same integrator were sampled taking cloacal swabs, drag swabs, and litter samples. These farms were sampled in two different seasons identified as pre-recommendations (March-April) and post-recommendations (October-November) periods. After confirmation of *Salmonella* with

PCR, all isolates were tested for antimicrobial resistance against 14 different antimicrobials using the Sensititre™ micro-dilution system and NARMS plates. All *Salmonella* isolates were further serotyped based on the antisera agglutination. Overall prevalence of *Salmonella* was 3-4% during March-April, while the prevalence ranged from 5-14% during October-November. Higher incidence of overall *Salmonella* for both seasons by sample type was observed in cloacal and drag sample with 5% for farm 1, drag swab with 6% in farm 2, cloacal swab with 6% for farm 3, and drag swab with 17% in farm 4. Differences in the prevalence of *Salmonella* were observed between the 2 seasons; however, these differences were not statistically significant ($p>0.05$). Based on these prevalence results, it can be inferred that irrespective of implementation of improved biosecurity practices, seasonal variation can cause changes in the prevalence of *Salmonella* on the farms.

Six different serotypes of *Salmonella* were identified with *S. Enteritidis* (52%) being the most prevalent, followed by *S. Berta* (38%), *S. Mbandaka* (7%), *S. Typhimurium* (2%), *S. Kentucky* (0.4%), and *S. Tennessee* (0.4%). A total of 7% isolates exhibited antimicrobial resistance to eight different antimicrobials. Higher resistance was observed for tetracycline, streptomycin, and nalidixic acid. A single isolate of *S. Mbandaka* exhibited multidrug resistance to tetracycline, amoxicillin/clavulanic acid, and ampicillin. Resistance to clinically important antimicrobials used to treat salmonellosis such as ceftriaxone, nalidixic acid, azithromycin, and ampicillin was observed which could be of particular concern to public health by reducing the options for treatment of foodborne illnesses.

3.2 Introduction

The US is the largest producer of poultry, and the second largest exporter of poultry meat in the world (USDA, 2012). *Salmonella* is an important pathogen highly associated with poultry products such as eggs and chicken meat (FSIS-USDA, 2016).

Antimicrobial resistance is a natural phenomenon that has been increasing over the years. Antimicrobial resistance is a mechanism that can be acquired or mutated (MacGowan & Macnaughton, 2013). When AMR is developed in bacteria, the susceptibility of bacteria to antimicrobials is lost, showing resistance to its effects, therefore their growth is not stopped and bacteria are not killed (Schwarz & Chaslus-Dancla, 2001). This resistance is increasing due to the misuse of antimicrobial agents in both human and veterinary medicine. Antimicrobial resistant bacteria can be transferred from animals to humans through contact with animals or through the food chain by consumption of products from food-producing animals colonized with this type of bacteria (Jindal et al., 2015).

One of the most important outbreaks of *Salmonella* in the US was the Foster Farms outbreak involving chickens contaminated with multidrug resistant (MDR) *Salmonella* Heidelberg. This outbreak occurred in 29 different states with 634 cases and a 38% hospitalization rate (CDC, 2014a). Seven different strains of *Salmonella* Heidelberg that were antimicrobial resistant (AMR) were found in the tainted chicken.

The National Antimicrobial Resistance Monitoring System (NARMS), tested 68 clinical isolates from ill people of which 65% were AMR and 35% were MDR. Different resistance patterns were exhibited by the clinical isolates, presenting combinations of ampicillin, chloramphenicol, gentamicin, kanamycin, streptomycin, sulfisoxazole, and tetracycline (CDC, 2014a).

Since chicken is the most consumed meat product in the US and outbreaks of *Salmonella* due to consumption of undercooked or contaminated poultry products are of high risk to human health (FSIS-USDA, 2016). In the US, foodborne illnesses due to *Salmonella* are the leading cause of hospitalizations and deaths, and the second highest cause of illnesses (FSIS-USDA, 2016).

The CDC reports that AMR poses an economic burden of \$35 billion to society because of a reduction on productivity and \$20 billion related to healthcare costs (CDC, 2013b) annually in the US. Foodborne illnesses caused by AMR bacteria result in longer stays at hospitals largely due to initial treatment failures (Hohmann, 2001) highlighting the need to address the issue.

In 2013, the USDA estimated a consumption of 57.7 pounds of chicken per person (ERS-USDA, 2015). Poultry products are often associated with *Salmonella* and the spread of *Salmonella* resistant to nalidixic acid, azithromycin, ampicillin and trimethoprim-sulfamethoxazole, which are important antimicrobial agents used for clinical treatments (CDC, 2015c) are of concern.

In order to fight increased AMR, the CDC has implemented different actions including the constant tracking of changes in AMR, providing recommendations to travelers regarding consumption of food and water, and educating healthcare centers among others (CDC, 2013b). Just like the CDC, different agencies such as the World Health Organization (WHO), have taken actions at a global level to fight AMR, beginning with The Global Strategy 2001 for AMR. The focus of this strategy is to take several measures such as education to patients, prescribers, and dispensers regarding implementation of antimicrobials and cooperation between industry, governments, and academic institutions to discover new drugs or vaccines (Jindal, 2015). Restrictions in the use of antimicrobials that are highly important for human medicine are also important. Strengthening regulations in veterinary medicine, intensifying collaborations between human and veterinary medicine, discovering new antimicrobials, and establishing surveillance systems for a prompt detection of AMR are some of the actions and initiatives taken from international entities in recent years (Paphitou, 2013). In response to the increasing prevalence of AMR bacteria, the CDC, FDA, USDA, and local and state health departments have created an interagency called National Antimicrobial

Resistance Monitoring System (NARMS). This interagency, is a surveillance system responsible for tracking AMR bacteria associated with raw meat and poultry, humans, and food producing animals (CDC, 2014a). This agency is focused on monitoring and analyzing changes in susceptibility to antimicrobials not only in *Salmonella* but other enteric bacteria. NARMS, tracks information for the CDC, FDA, and USDA regarding human illnesses, retail meats, and food animals, respectively in addition to collecting important information to establish regulations and generate reports regarding prevalence, changes, and statistics (CDC, 2015b).

3.3 Materials and Methods

3.3.1 Sampling Method

The Poultry Science department at Auburn University conducted a survey approved by the Auburn University Institutional Review Board of 120 broiler farmers from a single integrator to determine the management and biosecurity practices on each farm. Based on the response rate of the survey (27.5%; 32/120), four different poultry farms were selected for our research with similar characteristics such as ventilation, size, and number of broilers. All four farms were sampled for *Salmonella* in two different seasons; once before a series of biosecurity recommendations and a second sampling after the recommendations were made. These recommendations were made based on results from surveys taking into account lack of practices or programs such as rodent and insect control or supervision of visitors for both, houses and farms.

Based on the results from the survey, biosecurity practices that were not being followed were identified through the survey and recommendations were made. These recommendations consisted of visitors changing clothing before entering the farm, showering before and after entering the farm, maintaining records of entry and exit to farm, recording house entry, implementing rodent and insect control program, and the use of coveralls and shoe covers. Season one identified as “pre-recommendations season” covered the months of March and April while season two representing the “post-recommendations season” encompassed October and November. Each season had a total of 3 samplings, which were performed on day 1, day 14 and day 30 after the chicks were placed on the farms.

Drag swabs, litter and cloacal swab samples were collected in each farm. Each farm was composed of 4 houses, which were divided in 4 quadrants across the width so litter and drag samples could be taken in each of the quadrants. Twelve cloacal samples were taken from the center of the house. This resulted in a total of 16 drag swab samples, 16 litter samples, and 48 cloacal swab samples from each farm.

Sterile drag swabs (product no. DS-004; Solar-Cult, Ogdensburg, NY) were used to take drag swabs in each house. The first drag swab was dragged between the first water line and house wall starting from the middle of the house surrounding the water line to end at the middle of the house. This procedure was followed in the other half of the house surrounding the same water line to get a second sample. Samples three and

four were obtained by following the same procedure on the second water line.

Polyester tipped applicators (product no. 25-806 1PD; Puritan, Guilford, ME) were used to take cloacal swabs, which were pre-moistened with buffered peptone water (BPW; product no. C5323; Criterion, Santa Maria, CA). Samples were then inoculated into 5mL of BPW. Each litter sample was composed of three subsamples of 100g taken from different locations in the same quadrant of the house that were combined and mixed by hand.

After collection, all samples were processed at Auburn University. For enrichment, samples were added 20mL of Tetrathionate Brilliant Green Broth (TTB; product no. 95020-798; Himedia, Mumbai, India), vortexed and incubated at 37°C for 24h. One milliliter of BPW containing the cloacal swabs was added to 20mL of TTB and incubated at 37°C for 24h for *Salmonella* enrichment. Both drag and cloacal enrichments were streaked onto Xylose Lysine Tergitol-4 agar plates (XLT4; product no. 223420; BD, Sparks, MD) and incubated at 37°C for 48h.

Black colonies, indicating H₂S production, were subjected to precipitation test with antiserum (DIFCO™ *Salmonella* O Antiserum Poly A – I and Vi; product no. 222641, BD, Sparks, MD) for *Salmonella* confirmation. Furthermore, 90mL of Phosphate Buffered Saline (PBS) was added to 10g of litter sample in 15.2x22.9 cm filter bags (VWR Sterile Sampling Bag, product no. 89085-570, VWR), stomached for 60s and serially diluted, enriched, isolated and preserved similar to the swabs sample type (English, 2015).

Table 1. Characteristics of each farm sampled.

Farm Number	Ventilation Type	House Type	Brooding Arrangement	House Dimensions in feet (W×L)
1	Conventional	Curtain	Front End, Split ¹	40×400
2	Conventional	Curtain	Front End, Split ¹	40×400
3	Conventional	Solid Wall	Half House ²	40×500
4	Conventional (2), Tunnel (2)	Curtain	Front End, Split ¹	40×400

(English, 2015)

¹: Front half of the house was sectioned off for brooding, fencing from the middle to the front of the house along both interior water lines leaving an open walkway down the middle of the half of the house.

²: Front half of the house was sectioned off for brooding; chicks had full access to this section.

3.3.2 Reception of samples and cryopreservation

Isolates of presumptive *Salmonella* were shipped from the Department of Poultry Science at Auburn University to the Food Safety Laboratory in the Food Science Department at Purdue University in 1mL of Tryptic Soy Agar tubes containing a single isolated colony. Upon receiving, the colonies were grown in 10mL TSB (product no. 717164 B; Acumedaia; Lansing, MI) and incubated at 37°C for 24h. After incubation, each sample was streaked for isolation onto XLT4 plates and incubated at 37°C for 24h. A single isolated colony was taken from the XLT4 plates, based on typical *Salmonella* morphology, growth, and characteristics such as formation of black precipitate or black-centered colonies.

These isolated colonies were then grown on micro-centrifuge tubes (product no. 3213; Costar; Corning, NY) containing 1mL of TSB and incubated at 37°C for 24h. Following incubation, each colony was centrifuged at 5,000g for 3 min (Thermo Scientific; Model: Sorvall Legend Micro 17 Centrifuge). The supernatant was removed and the pellet was re-suspended in 1mL of 20% glycerol, which was finally mixed with sterile beads and cryopreserved at -80°C for further analysis.

3.3.3 DNA Extraction and quantification

For extraction of DNA, each sample was grown in TSB and incubated at 37°C for 24h. Following enrichment, each sample was streaked onto XLT4 agar plates and incubated at 37°C for 24h. As per the instructions from the National Veterinary Services Laboratory (NVSL) for serotyping, each isolate had to be confirmed as *Salmonella* before it was shipped. A single colony that was used for PCR confirmation targeting *hilA* gene was also sent for serotyping. Based on the morphology, black colonies were selected from the XLT4 agar plates, half of the colony was placed on 3mL of TSA for serotyping by the NVSL and the other half was used to inoculate 1mL of TSB in a micro-centrifuge tubes and incubated at 37°C for 24h. Boiling method was used to extract DNA based on Ngamwongsatit et al. (2008). After incubation, each micro-centrifuge tube was centrifuged at 5000 ×g for 2 min.

The supernatant was removed and the pellet was re-suspended in 500 μ L of DNase free water (product no. 10977-015, Life technologies; Grand Island, NY) and vortexed. Similar centrifugation step was repeated, the supernatant was discarded, and the pellet was re-suspended in 100 μ L of DNase free water. After vortexing, the micro-centrifuge tube was heated in a dry bath at 100 $^{\circ}$ C for 10 min. After boiling, the micro-centrifuge tube was centrifuged at 10,000 \times g for 5 min and the supernatant was removed and placed in sterile micro-centrifuge tube. An Epoch spectrophotometer (Biotek, Winooski, VT) was used for DNA quantification; 2 μ L of DNA from each isolate was placed in each cell. The panel was read in the spectrophotometer to determine the concentration and purity of DNA. The purity of the DNA was evaluated based on the protein/DNA ratio of absorbance (A260/A280) and the purity standard was a A260/A280 ratio between 1.8-2.0. Quantification of DNA was performed for results expecting concentration of 10 to 100ng, which was present in all the samples. After quantification, the DNA was stored at -20 $^{\circ}$ C until further use.

3.3.4 Polymerase Chain Reaction for *Salmonella* confirmation

DNA from each of the 595 isolate received from Auburn University were used to perform PCR and identify isolates positive for *Salmonella*. Forward and reverse primers and conditions for the reaction were used to target *hilA* gene for *Salmonella* identification. Based on Panthmanathan et al. (2003) *Salmonella* was detected by amplification of the *hilA* gene to select primers and as a base to set thermocycler conditions.

As forward primer, a 30bp primer (5'-CGGAACGTTATTTGCGCCATGCTGAGGTAG-3') and as reverse primer a 27bp primer (5'-GCATGGATCCCCGCCGCGAGATTGTG-3') (Sigma Life Science) were used to target *hilA* gene with a product of 784bp. For the reaction, GoTaq^R Green Master Mix (product no. M7122; Promega Corp., Madison, WI) with a concentration of 0.7X was mixed with a concentration of 0.5 μ M of each forward and reverse primers, DNA was diluted based on quantification results to obtain DNA template in concentration of 10-100 μ g and DNase free water to make the volume of 25 μ L for each reaction. For the negative control, DNase free water was used and for the positive control, DNA template of a known *Salmonella* strain (*Salmonella* Typhimurium ATCC 700720) was used. Reactions were performed with a QuantStudio (ThermoFisher Scientific) using different parameters with an initial denaturation cycle at 94 $^{\circ}$ C for 5 min., followed by a denaturation at 94 $^{\circ}$ C for 30s, an annealing temperature of 65 $^{\circ}$ C for 30s with 25 cycles and ending with an extension temperature of 72 $^{\circ}$ C for 10 min. After completion of each cycle, the reactions were held at 4 $^{\circ}$ C until use for gel electrophoresis. For gel electrophoresis, a 1% agarose gel with TBE buffer and 4 μ L of ethidium bromide with a concentration of 10mg/ml was used for product separation. A 100bp DNA ladder (product no. bp2571100; Fisher BioReagents; Canada) was placed in the 1st and 30th lanes of the gel which was run at 100v for 45 min.

3.3.5 Serotyping

All 262 confirmed *Salmonella* isolates were sent to the NVSL for serotyping. A sterile screw-cap 5mL tube (product no. C2540; Five-O) with 3mL of TSA (product no. 7100B; Acumedia; Lansing, MI) was inoculated with the isolate using a sterile loop and incubated at 37°C for 24h followed by storing the isolate at 4°C prior to sending it for serotyping. All TSA tubes containing single isolates were shipped to the NVSL on dry ice. *Salmonella* serotyping was based on agglutination for antisera where O antigens, H antigens phase 1, and H antigens phase 2 were used as a formula to identify serotypes based on Kauffman-White scheme.

3.3.6 Antimicrobial Resistance Testing

All positive *Salmonella* isolates were screened for antibiotic resistance. From each cryopreserved vials containing an isolated colony, a bead was streaked for isolation onto TSA plates using a sterile needle and incubated at 37°C for 24h. The procedure for Sensititre™ was followed based on instructions stated in the manufacturer's manual. A 5mL of Sensititre™ demineralized water (product no. T3339; Remel; Lenexa, KS) was inoculated with 3 to 5 colonies from the TSA plates using a sterile needle. The suspension was vortexed for visual comparison and adjustment to a 0.5 McFarland (product no. R20410; Remel; Lenexa, KS) polymer turbidity standard. Following this, 11mL of Sensititre™ cation adjusted Mueller-Hinton Broth (product no. T3462; Remel; Lenexa, KS) was inoculated by transferring 10µL of the suspension in order to have a final inoculum of 10⁵ CFU/mL. The tubes were then vortexed and its content was poured

into a sterile seed trough. Samples (50 μ L) of the inoculated broth in each well of the Sensititre™ Gram negative NARMS plate (product no. YCMV3AGNF; Remel; Lenexa, KS). The 96-well plate was covered with an adhesive seal and incubated at 36°C for 24h for further reading and analysis. Quality control was carried out during each testing day using *E. coli* ATCC 25922 as quality control, using same materials and following the same directions to determine AMR.

In order to read results from each plate, the quality control organism plate was read first, using CLSI specifications for this strain. To read the results of each plate, the positive wells were read first to make sure bacteria grew and after reading the positive wells, the negative wells were observed to identify any type of contamination. MIC were determined as the value of the well where no growth was observed. For trimethoprim and sulfamethoxazole well combination and the sulfisoxazole well, less than 10-20% growth compared to the positive cell, was considered as no growth, since these antimicrobials allow bacteria to grow before they are inhibited. Resistance was based in MIC breakpoints stated by the CLSI for *Enterobacteriaceae*, except for streptomycin since the CLSI does not have a recommendation; this value was obtained from recommendations by NARMS in the retail meat report (FDA, 2013). Multidrug resistance (MDR) was defined as resistance to more than 2 antimicrobial classes. Class antimicrobial categories were based on CLSI classification, which is presented in table 2. Plate format and concentrations for each antimicrobial are specified in figure 1.

SENSITITRE™ GRAM NEGATIVE NARMS PLATE FORMATPlate Code: **CMV3AGNF**

	1	2	3	4	5	6	7	8	9	10	11	12
A	FOX 32	AZI 8	CHL 16	AXO 64	AXO 0.25	CIP 2	GEN 16	NAL 16	XNL 2	FIS 32	AMP 32	STR 16
B	FOX 16	AZI 4	CHL 8	AXO 32	AUG2 32/16	CIP 1	GEN 8	NAL 8	XNL 1	FIS 16	AMP 16	STR 8
C	FOX 8	AZI 2	CHL 4	AXO 16	AUG2 16/8	CIP 0.5	GEN 4	NAL 4	XNL 0.5	SXT 4/76	AMP 8	STR 4
D	FOX 4	AZI 1	CHL 2	AXO 8	AUG2 8/4	CIP 0.25	GEN 2	NAL 2	XNL 0.25	SXT 2/38	AMP 4	STR 2
E	FOX 2	AZI 0.5	TET 32	AXO 4	AUG2 4/2	CIP 0.12	GEN 1	NAL 1	XNL 0.12	SXT 1/19	AMP 2	NEG
F	FOX 1	AZI 0.25	TET 16	AXO 2	AUG2 2/1	CIP 0.06	GEN 0.5	NAL 0.5	FIS 256	SXT 0.5/9.5	AMP 1	POS
G	FOX 0.5	AZI 0.12	TET 8	AXO 1	AUG2 1/0.5	CIP 0.03	GEN 0.25	XNL 8	FIS 128	SXT 0.25/4.75	STR 64	POS
H	AZI 16	CHL 32	TET 4	AXO 0.5	CIP 4	CIP 0.015	NAL 32	XNL 4	FIS 64	SXT 0.12/2.38	STR 32	POS

(ThermoFisher Scientific, 2016)

Figure 1. Sensititre Gram Negative NARMS panel, used for AMR test.

FOX= Cefoxitin; AZI= Azithromycin; CHL= Chloramphenicol; TET= Tetracycline;
 AXO=Ceftriaxone; AUG2= Amoxicillin/clavulanic acid 2:1 ratio; CIP= Ciprofloxacin;
 GEN=Gentamicin; NAL=Nalidixic Acid; XNL=Ceftiofur; FIS=Sulfisoxazole;
 SXT=Trimethoprim/ sulfamethoxazole; AMP= Ampicillin; STR= Streptomycin;
 NEG=Negative; POS= Positive.

Table 2. Antimicrobial agent classification for antimicrobial class and minimum inhibitory concentration (MIC).

Antimicrobial Class	Antimicrobial Agent	Resistance ($\mu\text{g/mL}$)
Cephems	Cefoxitin	≥ 32
	Ceftriaxone	≥ 4
	Ceftiofur	≥ 8
Macrolides	Azithromycin	≥ 32
Phenicols	Chloramphenicol	32
Tetracyclines	Tetracycline	≥ 16
Quinolones	Ciprofloxacin	≥ 1
	Nalidixic Acid	≥ 32
Aminoglycosides	Gentamicin	≥ 16
	Streptomycin	$\geq 64^*$
Folate Pathway Inhibitors	Sulfisoxazole	≤ 512
	Trimethoprim/Sulfamethoxazole	$\geq 4/76$
Penicillin	Ampicillin	≥ 32
	Amoxicillin/clavulanic acid	$\geq 32/16$

(CLSI, 2014)

Antimicrobial agents categorized under antimicrobial classes based on CLSI classification.

Resistance breakpoints based on CLSI breakpoints.

*: Value obtained from NARMS recommendation (USFDA, 2013).

3.3.7 Data Analysis

Salmonella prevalence was based on the number of positive samples obtained after each sampling. A sample was identified as positive when at least one isolate of the sample resulted positive for *Salmonella* after PCR confirmation. Antimicrobial resistant prevalence was determined as the proportion of isolates with resistance to at least one antimicrobial agent out of the total confirmed *Salmonella* isolates. Results of prevalence obtained during pre and post recommendation were compared in each farm to establish statistical significances between seasons. The same procedure was followed to establish differences in AMR prevalence between seasons for each farm. Since prevalence data was obtained in percentages (discrete dataset), an arcsine transformation was performed to obtain a continuous dataset and decrease variability (Warton and Hui, 2011). After data transformation, differences were analyzed using a comparative analysis using t-test where a p-value ≤ 0.05 was used to determined significant differences. All data was analyzed using the statistical program SAS 9.3 version.

3.4 Results and Discussion

3.4.1.1 *Salmonella* Prevalence

Table 3. Positive *Salmonella* isolates in each farm for both seasons.

Farm	Pre-Recommendations		Post-Recommendations		Total	
	No. Isolates	Positive Isolates	No. Isolates	Positive Isolates	No. Isolates	Positive Isolates
1	64	19 (30%)	95	26 (27%)	159	45 (28%)
2	30	12 (40%)	94	34 (36%)	124	46 (37%)
3	39	21 (54%)	50	35 (70%)	89	56 (63%)
4	112	10 (9%)	108	105 (97%)	220	115 (52%)

Presumptive *Salmonella* isolates from each sample were confirmed by PCR.

Results after PCR are shown in table 3, where 30, 40, 54, and 9% of the presumptive positive isolates for farm 1, 2, 3, and 4, respectively for the pre-recommendations season, were confirmed as *Salmonella*. Similarly, the percentage of positive isolates for the post-recommendations season were 28, 37, 63, and 52% for farm 1, 2, 3, and 4, respectively.

A decrease in the number of positive isolates after PCR confirmation was shown for all farms. Different annealing temperatures at 55, 58, and 62 °C were used in negative isolates to determine if this aspect was affecting the results. However, these lower values can be the result of false negatives because no universal primer or a different gene was targeted in this study.

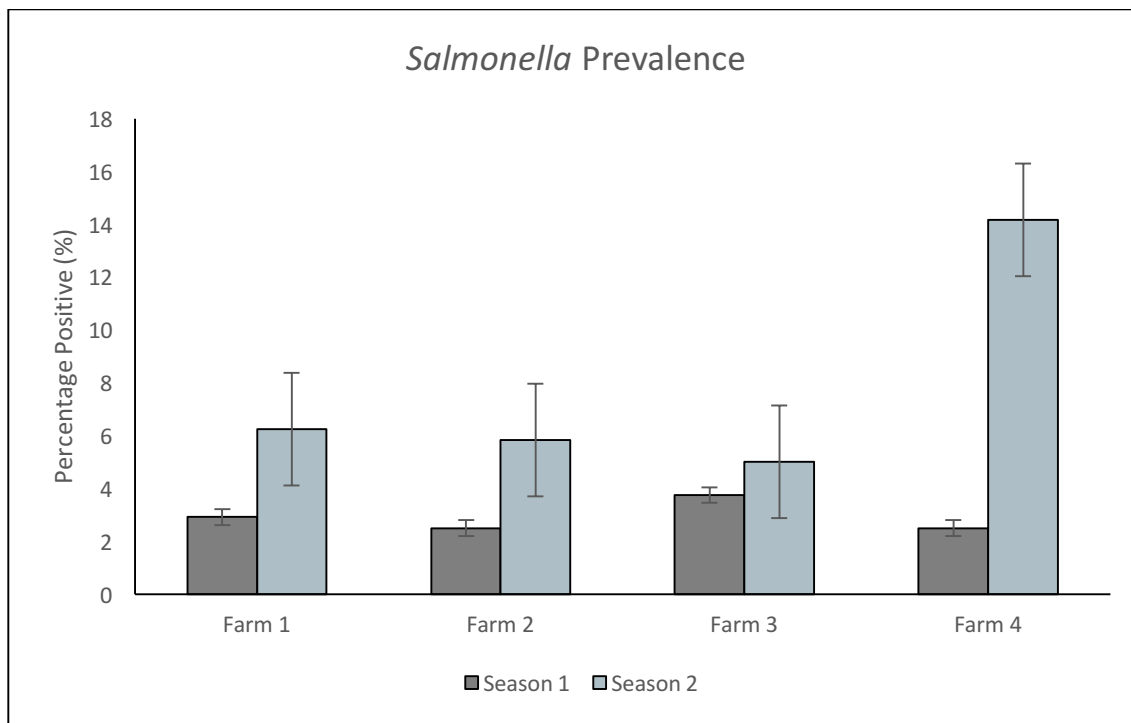


Figure 2. Prevalence of *Salmonella* among all four poultry farms during season 1 and 2.

Figure 2, presents the percentages of prevalence for both seasons. In season 1, prevalence of 3% for farm 1, 2, and 4, and 4% for farm 3 were observed. For season 2, a prevalence of 6% for farm 1 and 2, 5% for farm 3, and 14% for farm 4 were observed. No statistical significances ($p > 0.05$) were found when analyzing prevalence for both seasons (March-April and October-November respectively). When analyzing overall differences between both seasons for all four farms to determine if seasonality had an effect on the prevalence of *Salmonella*, significant differences ($p < 0.05$) were found, showing that season 2 (October-November) had a higher overall *Salmonella* prevalence rate of 4.32%.

Table 4. Prevalence of *Salmonella* by positive samples at house level.

Farm	Pre-recommendations	Post-recommendations
	Positive Houses (n=4)	Positive Houses(n=4)
1	3/4 (75%)	4/4 (100%)
2	3/4 (75%)	4/4 (100%)
3	3/4 (75%)	4/4 (100%)
4	3/4 (75%)	4/4 (100%)

Table 4, shows the prevalence of *Salmonella* at the house level within each farm. For prevalence of *Salmonella* measured by the presence of this pathogen at house level in each farm, positive results for *Salmonella* were present in three out of four houses on each farm with a 75% prevalence rate during the pre-recommendations season. Conversely, during the post-recommendations season the prevalence of *Salmonella* on all four farms was 100% in each house sampled. The overall prevalence at farm level was 100% in both seasons since *Salmonella* was present in each of the four farms.

In our study we observed 100% prevalence of *Salmonella* in both seasons on all four farms. Furthermore, 3 out of the 4 houses on each farm were positive for *Salmonella* at the pre-recommendation (March-April) phase of sampling, while all four houses on each farm were positive for *Salmonella* at the post-recommendation (October-November) phase of sampling. These results are consistent with prevalence values found in Alali et al. (2010), where *Salmonella* was present in all four broiler farms tested for *Salmonella* prevalence. On the other hand, the results of the present study are consistent with results from a study on newly organic farms conducted to

understand differences between conventional and organic farms. Prevalence of *Salmonella* at levels of 40% (2/5) in conventional farms and 100% (5/5) in newly organic farms were found; these levels of prevalence were based on positive farms (Sapkota et al., 2014). Results from the present study are also similar to prevalence in organic farms based on a study by Alali et al. (2010). By sampling one house in four conventional and three organic broiler farms of the same company in North Carolina, it was observed that *Salmonella* was present in 28.8% (115/400) and 4.3% (13/300) of the samples in conventional broiler farms and organic broiler farms, respectively (Alali et al., 2010). These prevalence rates appear higher than our study, as the researchers included broiler samples in their analysis.

Differences in prevalence between farms at the pre- and post-recommendations phase can be attributed to various aspects such as variation in seasons, possible irregular management of the farms, and differences in employees and their practices. Houses were cleaned out during spring, which could also explain the lower levels of *Salmonella* during the pre-recommendations season compared to the prevalence during post-recommendations season. Differences in the *Salmonella* prevalence in both seasons, can also be explained as a result of seasonal variation since it has been found that *Salmonella* prevalence can vary with each season during the year, being greater in fall than spring and summer (Bailey et al., 2001). *Salmonella* prevalence varies in all farms, ranging from 3% on farm 1, 2, and 4 in the March-April season and 14% in farm 4 during the October-November season.

Rectal samples obtained from broiler farms were observed to have varied *Salmonella* prevalence for different stages: for 5-day-old birds 8% *Salmonella* was found, for 20-day-old birds, a 13% prevalence was found, and a prevalence of 11.38% in adult broilers of 45 days was observed (Cui et al., 2016). In a study by Mathole et. al (2016), cloacal swabs were obtained from 286 chickens in South Africa, where 3.15% prevalence of *Salmonella* was reported. While geographic location is different, this rate of prevalence is similar to that found mostly for the pre-recommendations season in our study. Entry of pathogens into poultry and level of infections can be controlled via biosecurity practices and cleaning, mainly using all-in/all-out approach, as one of the most important source for contamination consist on sick animals (Ethelberg et al., 2014).

3.4.1.2 Biosecurity programs

Biosecurity programs are actions for preventing diseases or reducing the spread of pathogens that are critical to any agricultural production system. Due to poultry's association with *Salmonella*, it is important to manage and maintain practices that prevent this pathogen from entering into the production farms that can possibly have consequences later on in compromising the safety of the final products. While the implementation of biosecurity practices provides many benefits and prevents losses and severe consequences, not all poultry farmers implement these practices on their farms because of cost issues, lack of knowledge, and the involvement of training and administration process. Although farm biosecurity is important, sometimes it is

overlooked, since its implementation is not mandatory by state committees. Education and information to the farmers about this important aspect of production is needed in order to implement and maintain these programs. English (2015) conducted a survey on different poultry farms and found inconsistencies in the biosecurity practices implemented on the farms. The biosecurity improvements recommended here were based on the survey results from English (2015). Recommendations included change of clothing of visitors before entering the houses, shoe cover use, treatment of litter between flocks, implementation of rodent and insect control program, restricted entry of personnel, and registration of entry and exit to the farms.

According to the results presented in this study, the recommendation of biosecurity measures was not effective ($p > 0.05$) in minimizing the *Salmonella* prevalence on the farms. This can be attributed to several factors, such as seasonal variations and the variation in the implementation of the recommended biosecurity practices on different farms. The source of day-old chicks could have also played a role in; however, little information on the origin of the chicks was available. A survey on implementation of biosecurity practices was conducted on 61 poultry farms in Belgium with different aspects such as holdbacks to prevent diseases were analyzed. It was reported in this study by Laanen et. al (2014) that the increased cost for implementing biosecurity measures, along with the lack of rewards for producers and communication on how to implement biosecurity measures, were among the top 5 reasons for the lack of implementation of proposed biosecurity measures. However, there have been other

documented studies reporting that the cost of implementing biosecurity practices can be cost-effective in the longer term (Wegener et. al, 2003). This study was conducted in Denmark and it was reported that *Salmonella* controls resulted in an increase by \$0.02/Kg of broiler (Wegener et al., 2003). The cost of retail broiler is reported to be U.S. \$4.12/Kg (USDA, 2016). If the control practices recommended by the study in Denmark were applied, the cost to control *Salmonella* could result in a 0.5% increase of the retail price.

Based on practices to control *Salmonella* on broiler farms in Denmark, industrywide programs were initiated, which were successful in decreasing *Salmonella* in broilers. This program consists of eradicating *Salmonella* from the beginning of the value chain by eliminating infected breeding animals and giving farmers a better price for *Salmonella* free birds. Intensive testing in flocks and removing contaminated animals were found to eradicate *Salmonella* in broilers (Wegener et al., 2003). Informing the producer about the cost and the benefits can have a positive impact on the perspective that farmers can get and therefore facilitating the process of implementation.

As evident, implementation of biosecurity practices in poultry are important for prevention or control of pathogens. One of the recommendations that was made in our study was rodent control which is essential, since rats can contaminate disinfected houses with *Salmonella* specifically (Lister 2008). Recommendations on hygiene and disinfection were not made, however Wales et al. (2006) demonstrated that while

Salmonella eradication was not possible, there was a decrease in the prevalence of this pathogen after dry cleaning. Spread of pathogens between farms can take place when farms are close to each other, and also through vectors such as insects, rodents, and birds as well as humans (Albihn and Vinnerås, 2007). *Salmonella* can be transmitted through cockroaches, which can act as a vector to transmit this pathogen (Keener, 2011). Improvement of insect control can reduce entry of pathogens into the houses if they are implemented correctly.

3.4.2 *Salmonella* Serotypes

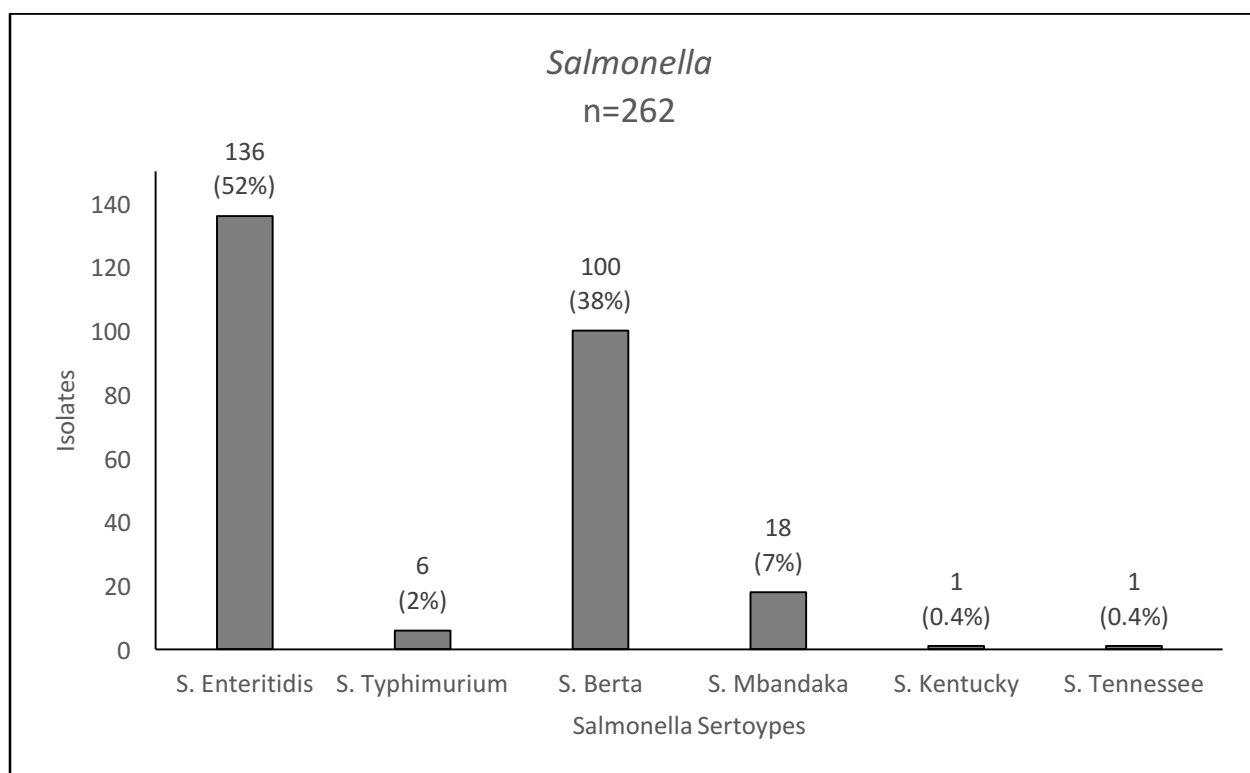


Figure 3. Distribution of *Salmonella* serotypes found in all four poultry farms.

Figure 3, represents the total distribution of *Salmonella* isolates found in all four farms.

Among all 262 confirmed *Salmonella* isolates, 6 different serotypes were identified, these being *S. Enteritidis*, *S. Typhimurium*, *S. Berta*, *S. Mbandaka*, *S. Kentucky*, and *S. Tennessee*. Among all 262 *Salmonella* isolates, the most common serotype was *S. Enteritidis* with 52% (136/262), followed by *S. Berta* 38% (100/262), *S. Mbandaka* 7% (18/262), *S. Typhimurium* 2% (6/262), and the remaining 1% of *S. Kentucky* and *S. Tennessee* with one isolate of each serotype.

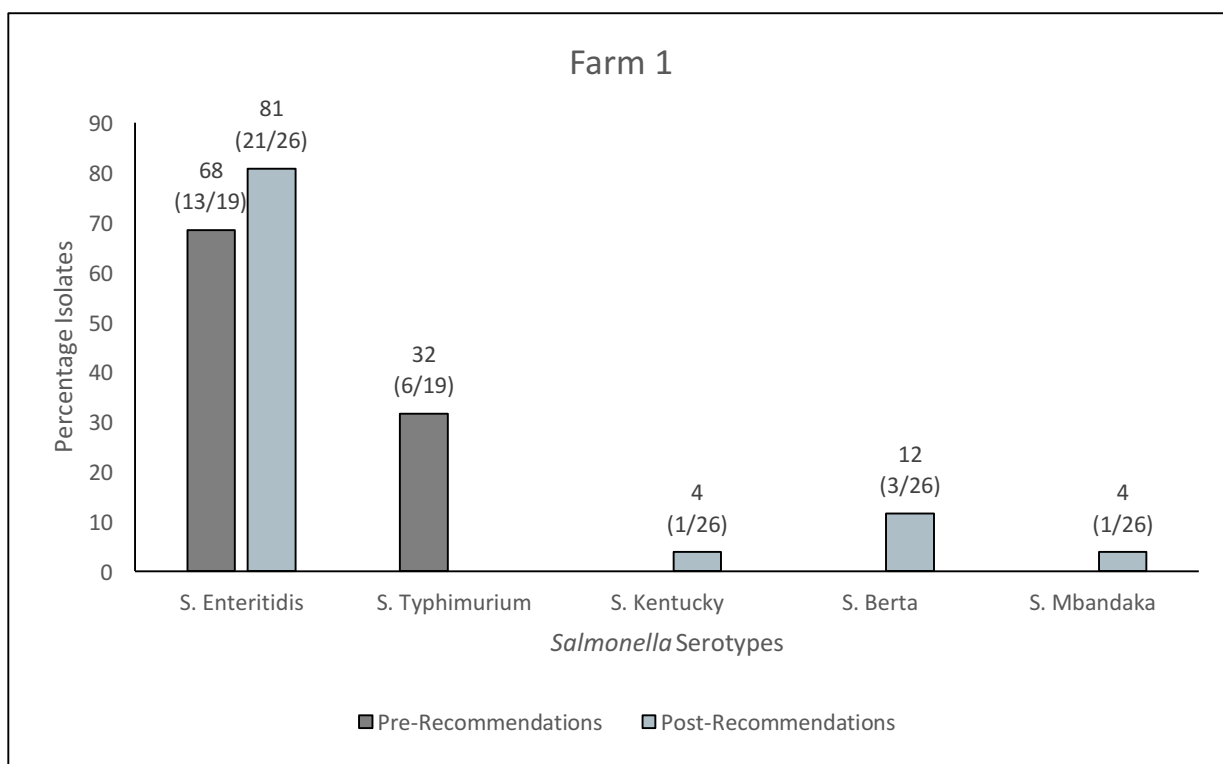


Figure 4. Distribution of *Salmonella* serotypes found in farm 1 in both seasons.

On farm 1 (figure 4), *S. Enteritidis* was observed to be the most common serotype in the first season, followed by *S. Typhimurium*. These serotypes are the only two serotypes found in both first season and in the second season, new serotypes were found with lower prevalence than *S. Enteritidis* as this remains the most prevalent serotype in the second season also. In comparison to the other farms, farm 1 showed a higher diversity of serotypes, having 5 different serotypes out of the total 6 found on all farms. *S. Enteritidis* differs from *S. Typhimurium* in that the first one is present in both seasons, whereas the latter one is only present in the first season but not in the second season.

Besides *S. Enteritidis* and *S. Typhimurium* in the second season, *S. Berta* is present in the post-recommendations season followed by *S. Mbandaka*, and *S. Kentucky*.

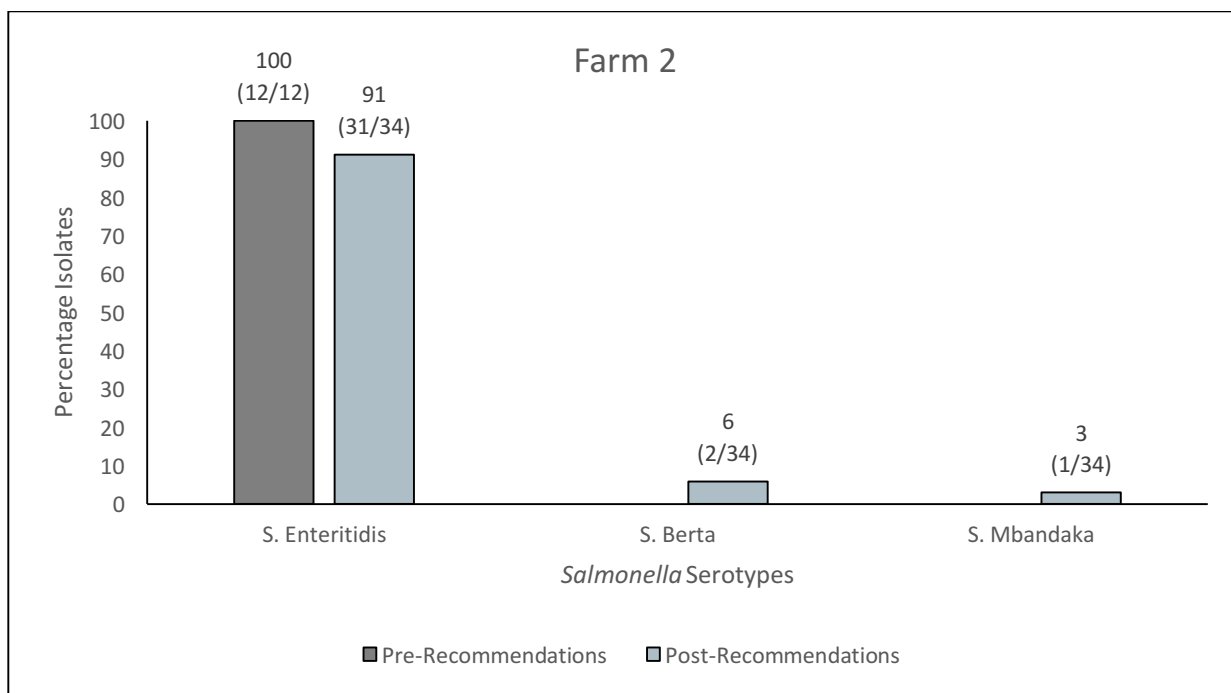


Figure 5. Distribution of *Salmonella* serotypes found in farm 2 in both seasons.

For farm 2 (figure 5) during the pre-recommendations season, the only serotype present is *S. Enteritidis*, which is also the most prevalent serotype in the post-recommendations season followed by *S. Berta* and *S. Mbandaka*.

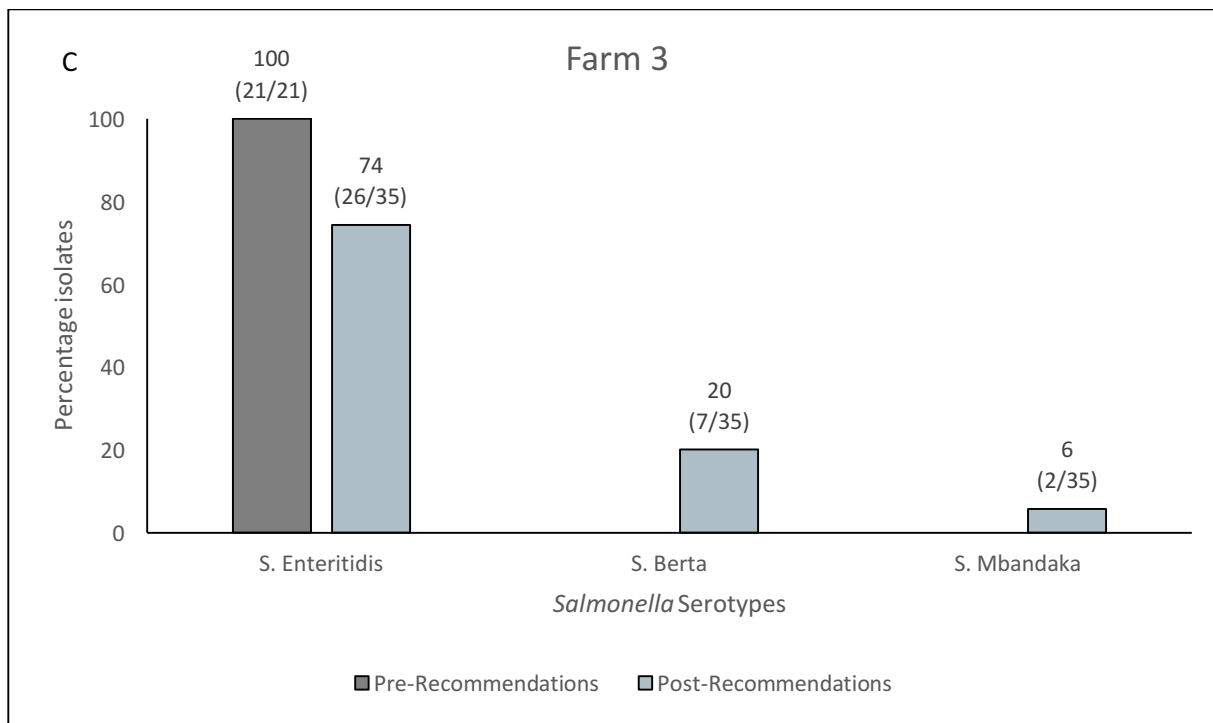


Figure 6. Distribution of *Salmonella* serotypes found in farm 3 in both seasons.

Farm 3 (figure 6) showed similar behavior to farm 2, where *S. Enteritidis* is the only serotype present in the pre-recommendations season. This serotype became the most prevalent one whereas, *S. Berta* and *S. Mbandaka* appear during the post-recommendations season with *S. Berta* being the second most prevalent followed by *S. Mbandaka*.

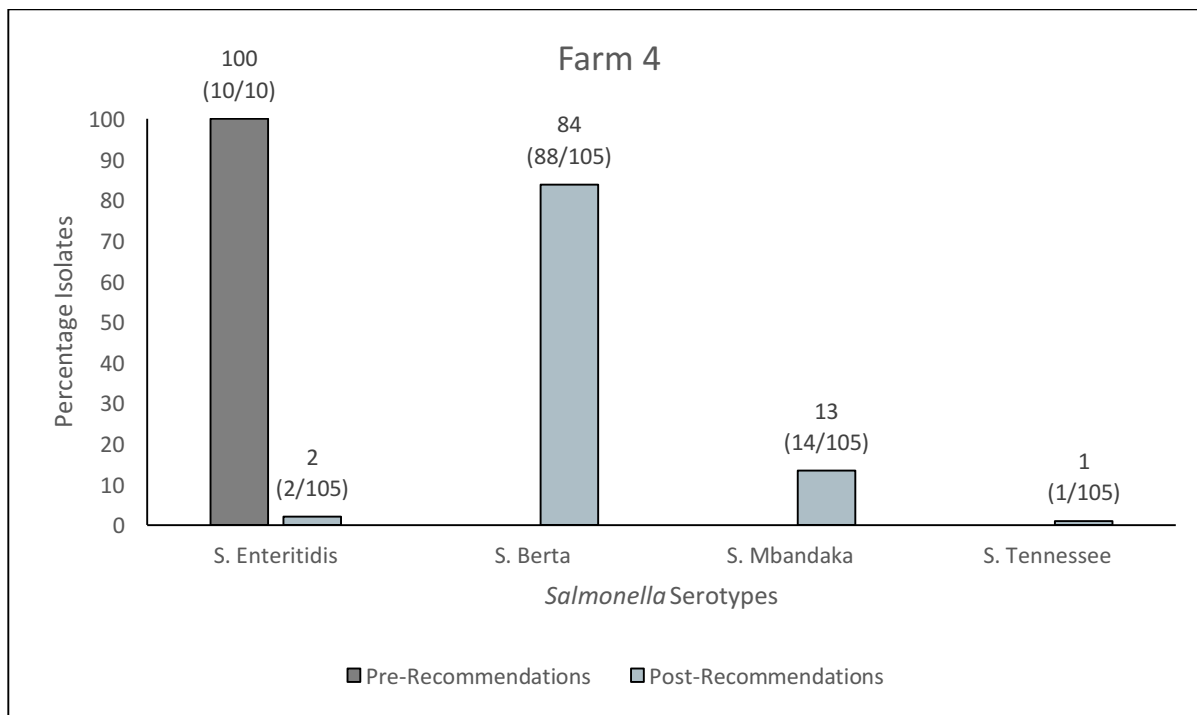


Figure 7. Distribution of *Salmonella* serotypes found in farm 4 in both seasons.

Unlike the other farms, farm 4 (figure 7) is mostly associated with *S. Berta* during the post-recommendations season. *S. Enteritidis*, *S. Mbandaka* and *S. Tennessee* are present as well. The only *S. Tennessee* found among all *Salmonella* isolates is found in the second season on this farm.

After *S. Enteritidis*, the most prevalent serotype found was *S. Berta* which was also found in all four farms, but majority of *S. Berta* was found on farm 4 (figure 7) for the post-recommendations season. Given the known and well documented information that *S. Enteritidis* is the most common serotype found on poultry farms, mitigation efforts are usually focused on this serotype, however, from our study it is evident that

S. Berta is the most prevalent serotype on farm four and there is a possibility that these mitigation strategies might not be effective against other highly prevalent *Salmonella* serotypes. When analyzing overall serotypes prevalence, taking into account the prevalence in both seasons, the most common serotype found in farm 1, 2, and 3 was *S. Enteritidis* with 76, 93, and 84% prevalence, respectively. The exception to this serotype prevalence was observed on farm 4, where *S. Berta* was found to be the most prevalent serotype with an overall prevalence of 77%. *S. Enteritidis* is the only serotype present in the pre-recommendations season, and it is during post-recommendations season when other serotypes are found. With the exception to *S. Typhimurium* on farm 1 besides *S. Enteritidis* during the pre-recommendations season and it is no longer found during post-recommendations season.

Given the inconsistencies in the prevalence rates and serotypes on different farms, it can be concluded that controlling *Salmonella* is a challenging task and developing biosecurity measures that can control all serotypes on *Salmonella* on the farm is not possible to implement.

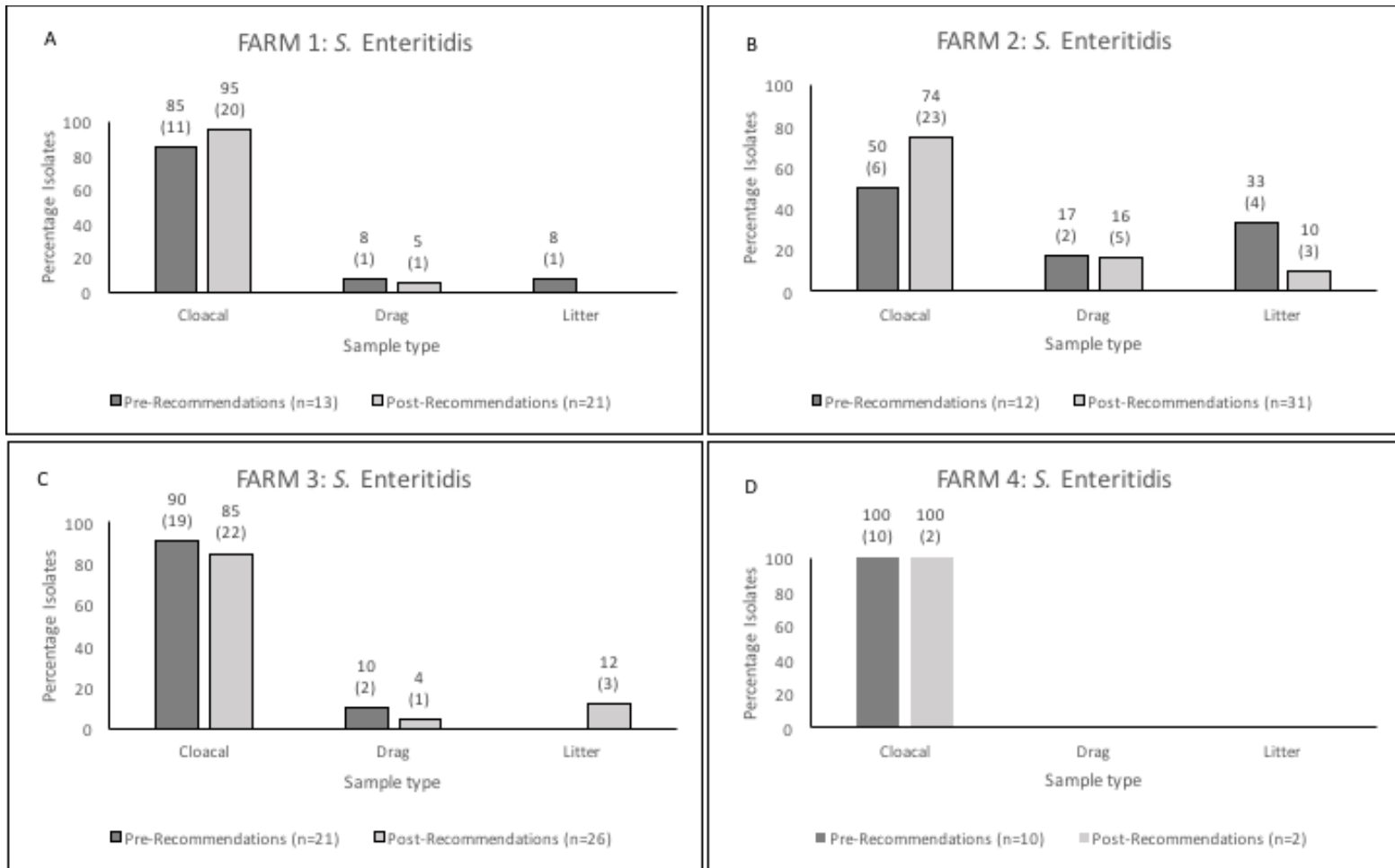


Figure 8. Incidence of *S. Enteritidis* by sample type in each farm for both seasons. A=Farm 1; B=Farm 2; C=Farm 3; D=Farm 4.

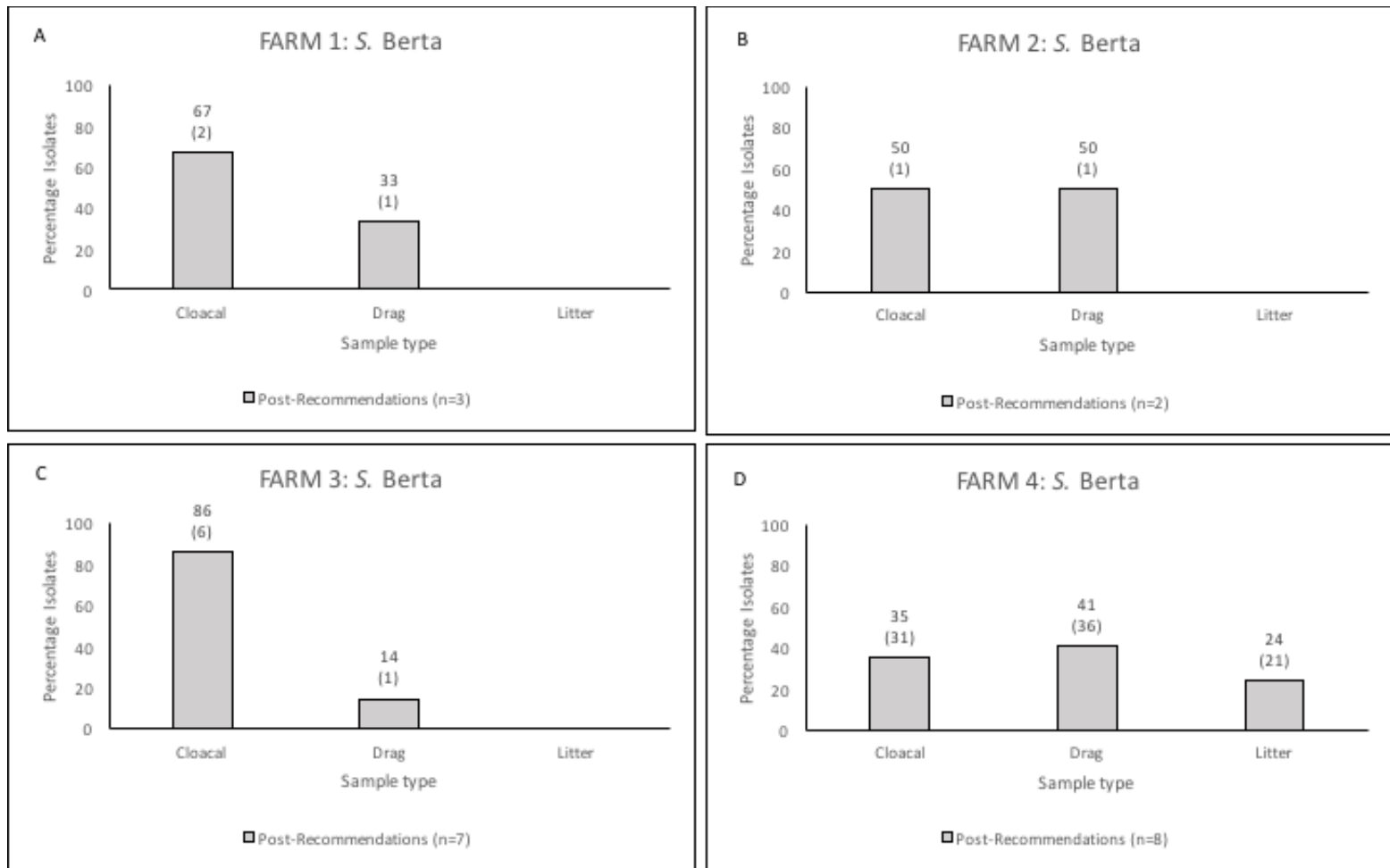


Figure 9. Incidence of *S. Berta* by sample type in each farm for both seasons. A=Farm 1; B=Farm 2; C=Farm 3; D=Farm 4.

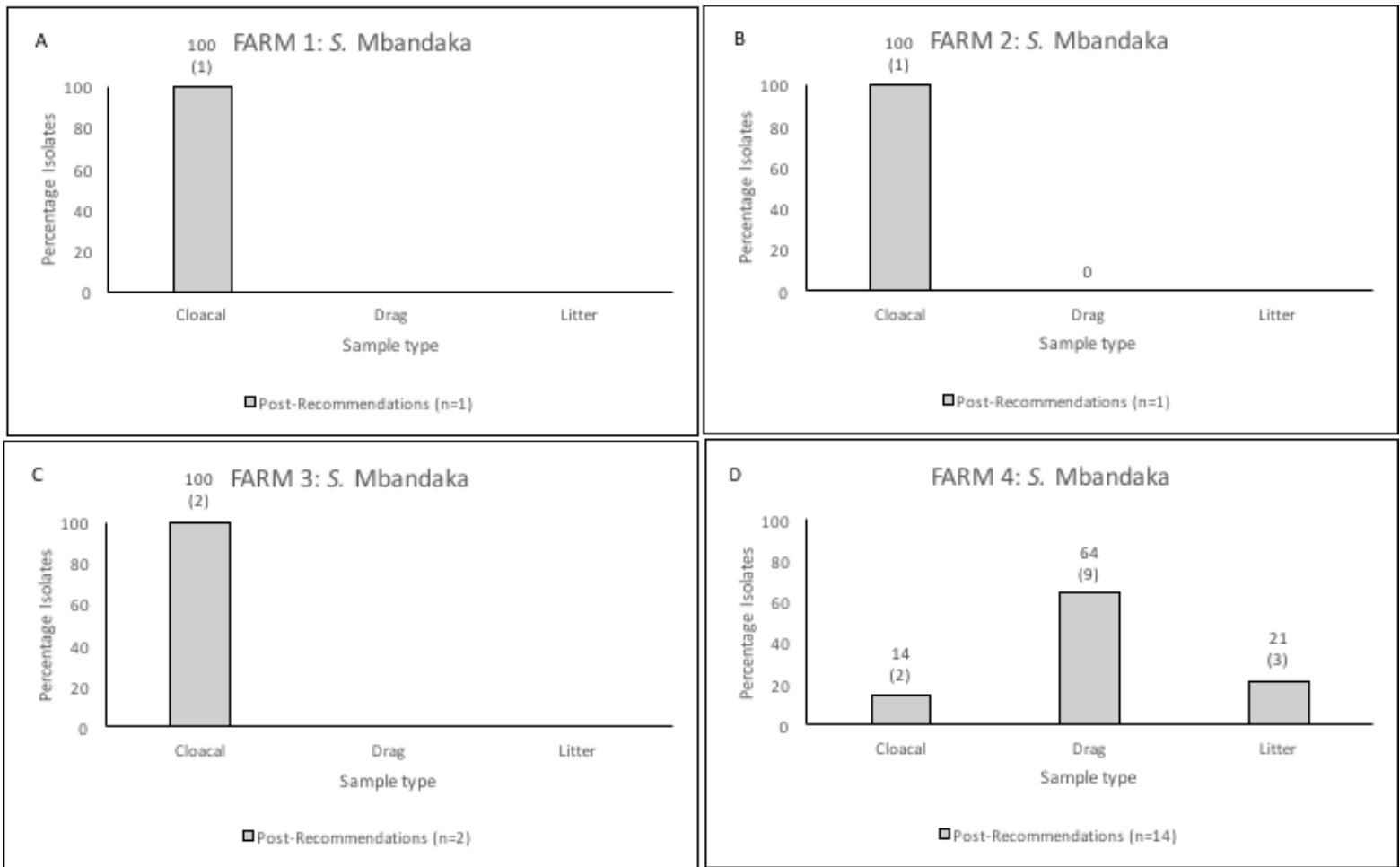


Figure 10. Incidence of *S. Mbandaka* by sample type in each farm for both seasons. A=Farm 1; B=Farm 2; C=Farm 3; D=Farm 4.

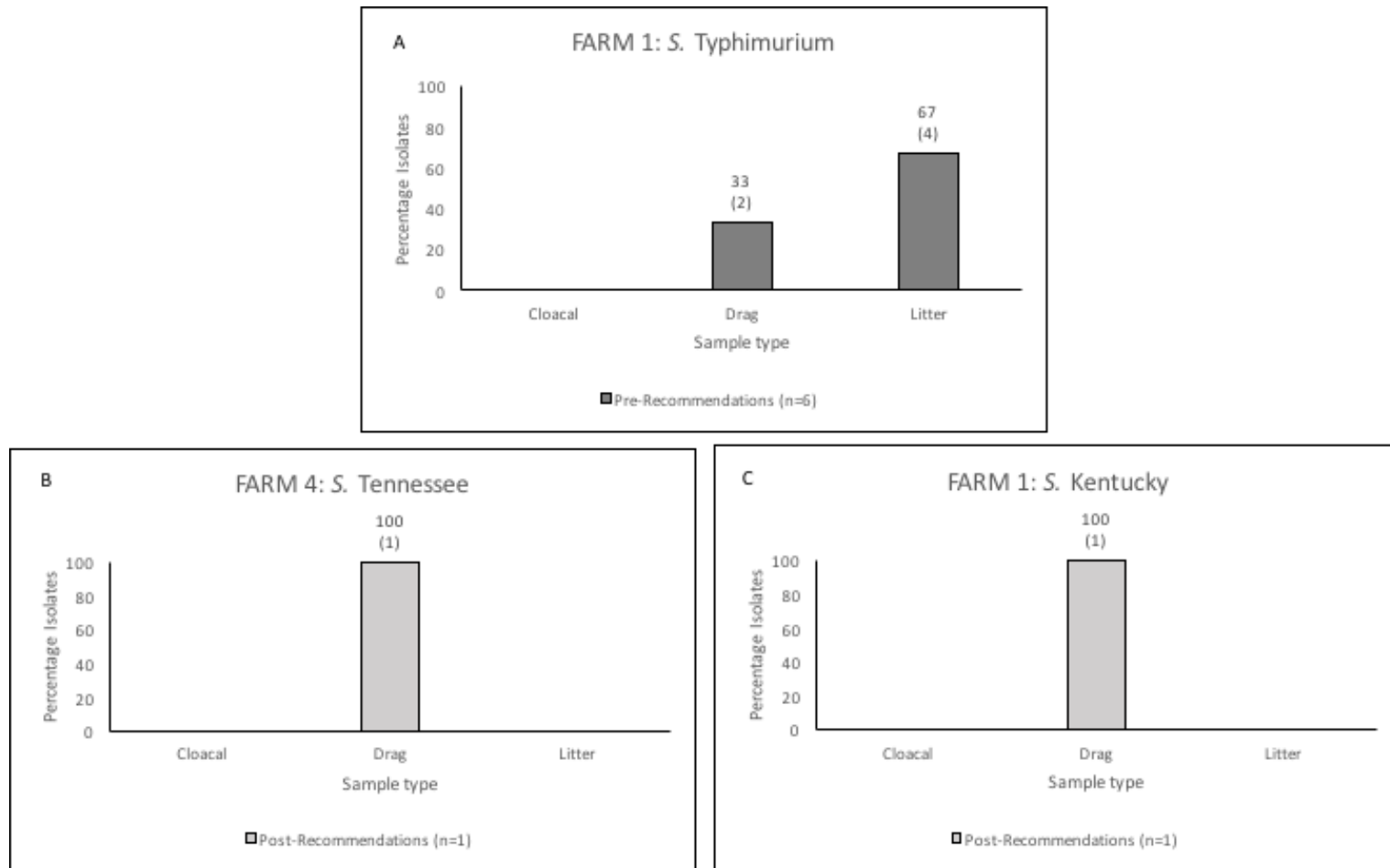


Figure 11. Incidence of the least common serotypes by sample type. A= *S. Typhimurium*; B= *S. Tennessee*; C= *S. Kentucky*.

S. Enteritidis was one of the serotypes present in all four farms. By dividing the incidence of this serotype in sample type on each of the four farms, it was found that the sample with a higher incidence of this serotype was cloacal samples. As shown in figure 8, *S. Enteritidis* is present in the cloacal swab samples on all four farms sampled in our study during the pre and post-recommendations season. For the drag swab samples, this serotype is present in both seasons on farms 1, 2, and 3. On the other hand on farm 4, this serotype is not associated with drag swabs. For litter samples, farm 2 is the only one where this serotype was present in both seasons. On farm 1, this serotype is present in litter samples only during the pre-recommendations season, different from farm 3, where this serotype occurs only during post-recommendations season.

In our study, *S. Berta* was observed to be the second most prevalent serotype, on all four farms. This serotype was found in the litter samples only on farm 4 during the post-recommendations season. As shown in figure 9, this serotype is associated with cloacal and drag swabs samples for farm 1, 2, and 3; farm 4, is the only farm where it can be observed that this serotype is associated with all the three sample types. Figure 10 shows the distribution of *S. Mbandaka* on all farms only during the post-recommendations season. It can be observed that cloacal swab samples are the only sample type from where this serotype was isolated. For farm 4, this serotype is associated with all sample types.

From figures 8, 9, and 10 it can be observed that farms 1, 2, and 3 follow the same trend based on the distribution of each serotype with sample type. Whereas farm 4, is the only one that behaves differently, since it is the only one from where *S. Berta* and *S. Mbandaka* were associated with all the three sample types. Similarly, farm 4 behaves differently from the rest with respect to *S. Enteritidis*, where cloacal swabs were the only sample type associated with this serotype. Figure 11 shows the distribution of *Salmonella* serotypes found in only one farm. As shown on figure A, *Salmonella* Typhimurium was found during pre-recommendations season only in farm 1 in drag and litter sample, where the latter one had the higher prevalence. On the other hand on figure B and C, a single isolate of *S. Tennessee* and *S. Kentucky* were found in drag sample during post-recommendations season.

This is different to what was reported by Singh et al. (2013), where *S. Typhimurium* was isolated from cloacal samples with a prevalence of 4.4% (8/180) in layer chicken. On the other hand, *S. Tennessee* which, was present only on farm 4 and *S. Kentucky* on farm 1. These single isolates were both present only in the post-recommendations season and associated exclusively with drag swabs. Three out of the six serotypes found in this study, are in the list of the top 20 most common serotypes associated with public health based on the CDC report from 2011, namely *S. Enteritidis* (1/20) with 16.5% infections, *S. Typhimurium* (2/20) with 13.4% infections, and *S. Berta* (19/20) with the lower percentage of infections with 0.7% (CDC, 2011). NARMS reports distribution of *Salmonella* serotypes found in retail chicken, with the most prevalent

serotype being *S. Typhimurium* with 43% (66/158), *S. Enteritidis* was present in 13% (21/158) of the samples, *S. Kentucky* was isolated from 28% (45/158) samples and *S. Mbandaka* from 2% (3/158) samples. No *S. Berta* was found in retail chicken meat samples (FDA, 2013).

Based on the CDC Atlas for *Salmonella* isolated from humans from 1968 to 2011, *S. Berta*, *S. Enteritidis*, *S. Mbandaka*, and *S. Typhimurium* were isolated from humans. *S. Berta* was associated with chicken, 52% (577/1305) for clinical sources and 65% (810/1246) of non-clinical sources; *S. Enteritidis* with a 50% (15526/30880) incidence on chicken from clinical sources and 83% (5513/6677) for non-clinical sources; *S. Mbandaka* was isolated from chicken 30% (1660/5484) clinical sources and 49% (1248/2571) of non-clinical sources. All the above-mentioned serotypes had higher percentages of isolation from chicken in both clinical and non-clinical sources. The exception to this was *S. Typhimurium*, which had chicken as the fourth source and not as the main source compared to the other serotypes. *S. Typhimurium* was isolated from 10% (4941/49455) clinical sources from chicken, and with 29% (1893/6476) from non-clinical sources from chicken being this the main source (CDC, 2013a).

Poultry farms were sampled obtaining fecal and environmental samples using drag swabs. The most prevalent serotypes were *S. Havana* and *S. Enteritidis* in broilers. *S. Mbandaka* was identified in 42% (5/25) broilers (Clemente et al., 2015). In Portugal there has been an increase association of *S. Mbandaka* with broilers over the last six

years (Clemente et al., 2015). Being *S. Mbandaka* found in broilers in countries different than the U.S. suggests that this serotype is adapted to this production system therefore prevalence values of this serotype can increase if it is not controlled. By knowing the actual serotypes that are present on the farms is important to create control programs to decrease or eliminate the presence of that specific serotype. Creating actions plans to *control S. Typhimurium* can be done such as decreasing the pH of litter. It has been demonstrated by Soliman et al., (2009) that this change on pH decreases the survival of this serotype.

Litter is a source of *Salmonella* since it has been demonstrated that fresh litter was contaminated with *Salmonella* (Bhatia et al., 1979). *Salmonella* control such as vaccination to fight specific serotypes can be achieved as demonstrated in Young et al. (2007), where a decrease of 28% in *S. Kentucky* was seen when challenging birds with this serotype in 1-day-old birds. Classification of *Salmonella* serotypes is based on the surface due to different chemical composition and differences on flagella which can be differentiated by concentration of protein (CDC, 2015d). By identifying serotypes and composition of their structure can be critical to monitor these serotypes and have a narrow action plan.

Ten different serotypes including *S. Enteritidis* and *S. Typhimurium* were tested to determine ability of biofilms formation. Biofilm formation was not seen only at serotype level, but at strain level. In the case of *S. Typhimurium* 50% were classified as moderate, whereas *S. Enteritidis* had 59.5% of strains as weak biofilm producer (Díez-Gracia et al., 2012). If differences on the biofilm formation can be studied to all the serotypes found in this study, control of these pathogens can be achieved, either by use of specific chemicals or disinfectants that will be effective based on their mechanism of action against specific serotypes. In Díez-Gracia et al. (2012) was observed that *S. Enteritidis* was able to form biofilm on plastic surfaces, therefore this approach can be used to change tools that are being used on the farms to avoid cross contamination between houses.

Findings on these serotypes are similar in serotypes found in Roy et al. (2002) in that *S. Kentucky*, *S. Enteritidis*, *S. Mbandaka*, *S. Berta* and *S. Enteritidis* and *S. Typhimurium* were isolated from poultry, poultry products, and poultry environment.

3.4.3 Antimicrobial Resistance

Table 5. *Salmonella* isolates with antimicrobial resistance found in all four farms.

Farm	Pre-Recommendations	Post-Recommendations
	No. Positive Isolates	No. Positive Isolates
1	3/19 (16%) ^A	4/26 (15%) ^A
2	1/12 (8%) ^A	1/34 (3%) ^A
3	1/21 (5%) ^A	2/35 (6%) ^A
4	1/10 (10%) ^A	6/105 (6%) ^A

^A: Same letter represent no significant differences between season ($p > 0.05$).

All isolates positive for *Salmonella* were subjected to AMR testing. From the 262 *Salmonella* isolates tested against 14 different antimicrobials, as shown in table 5, a total of 19 isolates exhibited resistance to at least one antimicrobial agent, which represents 7% (19/262) of all isolates.

Table 6. Antimicrobial resistance patterns in *Salmonella* isolates.

Resistance Pattern	Sample Type	Serotype
TET+AUG+AMP	Litter	S. Mbandaka
TET+STR	Drag	S. Kentucky
TET+STR	Cloacal	S. Berta
TET+STR	Drag	S. Berta
AXO+NAL	Cloacal	S. Enteritidis
AZI+TET	Cloacal	S. Enteritidis
TET	Cloacal	S. Enteritidis
TET	Cloacal	S. Enteritidis
TET	Drag	S. Enteritidis
TET	Cloacal	S. Berta
TET	Drag	S. Berta
TET	Drag	S. Berta
TET	Cloacal	S. Mbandaka
NAL	Litter	S. Enteritidis
NAL	Cloacal	S. Enteritidis
NAL	Drag	S. Enteritidis
STR	Cloacal	S. Berta
STR	Drag	S. Typhimurium
CHL	Drag	S. Berta

TET = Tetracycline; AUG = Amoxicillin Clavulanate; AMP = Ampicillin; STR = Streptomycin; AXO = Ceftriaxone; NAL = Nalidixic Acid; AZI = Azithromycin; CHL = Chloramphenicol.

Table 6, presents each characteristic of the resistant isolates, resistance patterns, sample type, and serotype. It can be observed that all four farms presented different characteristics regarding resistance patterns since different patterns were observed in each farm, there was no correlation found between AMR pattern and serotype as well as sample type. It was observed that 63% (12/19) of the isolates were resistant to tetracycline, followed by 26% (5/19) being resistant to streptomycin and 21% (4/19) being resistant to nalidixic acid. In contrast, lower resistance (5%; 1/19) was observed for ceftriaxone, azithromycin, chloramphenicol, ampicillin and amoxicillin/clavulanic acid. Out of the 14 antimicrobials tested for resistance, all *Salmonella* isolates were susceptible to 6 antimicrobial agents, namely, ceftiofur, ciprofloxacin, gentamicin, sulfisoxazole, and trimethoprim/sulfamethoxazole. Out of all AMR *Salmonella*, 68% (13/19) were resistant to at least one antimicrobial class, 26% (5/13) were resistant to 2 classes and 5% (1/19) were resistant to 3 antimicrobial classes, which was classified as multidrug resistant (MDR). From the 13 isolates with resistance to one antimicrobial class, 54% (7/13) were resistant to tetracycline which belongs to the tetracycline antimicrobial class, 23% (3/13) resistant to nalidixic acid from quinolones class, 15% (2/13) resistant to streptomycin from aminoglycosides class, and the remaining 8% (1/13) were resistance to chloramphenicol which belongs to the phenicols antimicrobial class. A total of 26% (5/19) were resistant to two classes of antimicrobials, from which 60% (3/5) showed resistance against streptomycin+tetracycline belonging to the streptomycin and tetracycline class, respectively. The rest of the patterns were distributed equally where 20% (1/5) had a ceftriaxone+nalidixic acid resistance pattern

belonging to the cepheems and quinolones class respectively, and the other 20% (1/5) were resistant to azithromycin+tetracycline representing macrolides and tetracycline class, respectively. The only MDR isolate exhibited a tetracycline+amoxicillin/clavulanic acid+ampicillin resistance pattern belonging to the tetracycline class, betalactams and penicillin class, respectively. The most prevalent serotype among the resistant *Salmonella* was *S. Enteritidis* with 42% (8/19), followed by 37% *S. Berta* (7/19), *S. Mbandaka* with 11% (2/19) and 5% (1/19) for each *S. Typhimurium* and *S. Kentucky*. When classifying resistance according to antimicrobial classes, the most prevalent serotype among resistant isolates was *S. Enteritidis*, resistant to one antimicrobial class. Conversely, *S. Berta* was the most common serotype with resistance to two classes of antimicrobials and the only MDR isolate was identified as *S. Mbandaka*. Cloacal samples were most often antimicrobial resistant (47%; 9/19), followed by 42%(8/19) drag swabs and only 11% (2/19) associated with litter sample from which one was the MDR isolate.

From conventional farms, it was found that 36.2% (21/58) exhibited resistance to only one antimicrobial agent, and 62% (36/58) were resistant to at least two antimicrobial agents (Alali et al. 2010). In that study, there was 6.9% (4/58) of isolates resistant to tetracycline, 91.4% (53/58) resistant to streptomycin, 55.2% (32/58) were resistant to amoxicillin/clavulanic acid, and 56.9% (33/58) resistant to ampicillin. All isolates subjected to AMR test were susceptible to ceftriaxone, nalidixic acid, and chloramphenicol (Alali et al., 2010). In Singh et al. (2013), only 26 isolates were tested against antimicrobial resistance. From these isolates, however, 23.08% (6/23) was

found to be resistant to tetracycline followed by 23.08% (6/26) resistant to chloramphenicol and 11.5% (3/26) resistant to streptomycin. A study conducted in retail chicken carcasses found a 34% of *Salmonella* prevalence rate based on PCR confirmation from which the most common serotype was *S. Typhimurium* with 44.1% (30/68). Resistance to tetracycline was present in 67.6% (46/68) isolates, followed by 61.7% (42/68), and 10.2% (7/68) resistant to chloramphenicol and 100% resistance to penicillin (Yildirim et al., 2011). These differences in the prevalence of AMR isolates can be attributed to the number of isolates tested as well as the selection process. In the present study, all the PCR confirmed *Salmonella* were subjected to antimicrobials susceptibility in contrast with Alali et al. (2010) study, where 58 isolates out of 115 isolates were tested against AMR. This represents 50% versus 100% of all the *Salmonella* present in conventional farms. In litter samples from organic poultry farms, 17% (1/16) isolates of *S. Enteritidis* exhibited resistance to tetracycline. It was found that *S. Kentucky* was the most common serotype, and 44% (4/9) of these isolates exhibited a resistance pattern to streptomycin and tetracycline. *S. Mbandaka* showed resistance most commonly to tetracycline and streptomycin. Resistance to quinolones was observed in *S. Mbandaka* isolated from poultry (Hoszowski and Wasyl, 2001). This resistance to quinolones observed in *S. Mbandaka* presents a special public health threat as the presence of this pathogen in broilers is not commonly observed, therefore eradication of *Salmonella* is not typically focused on this serotype. Cepheids are third generation extended spectrum cephalosporins antimicrobials and are commonly used to

treat *Salmonella* infections. There was no resistance found to any of these antimicrobial agents of this class such as ceftriaxone, ceftiofur, and ceftiofur (Sapkota et al., 2014).

One isolate out of the 19 antimicrobial resistant isolates showed resistance to tetracycline and azithromycin. This latter antimicrobial is used to treat severe *Campylobacter* infections (Mølbak, 2005). In a limited basis, the use of azithromycin to treat *Salmonella* infections has been studied, suggesting this antimicrobial agent can be used as an alternate antimicrobial to treat these infections (Hohman, 2001). Nalidixic acid is categorized as a quinolone, an antimicrobial class that is known to be one of the first choice antimicrobials to treat salmonellosis. Resistance in 23% of the isolates to this antimicrobial agent is concerning, due to the importance that these antimicrobials represent in human medicine. Isolates with resistance to this antimicrobial agent are likely to develop resistance to other antimicrobial agents that also belong to the quinolones class (Sárközy, 2001). A resistance pattern to nalidixic acid and ceftriaxone was seen in one isolate. This cross-resistance can be attributed to mutations that occur when resistance to fluoroquinolones is present as mutations conferring cephalosporins and tetracyclines resistance have been reported (Sárközy, 2001). When bacterial strains exhibit resistance to an antimicrobial agent of any particular antimicrobial class, resistance to other antimicrobial agents from that same class are likely to occur (Tenover, 2006). In this study, the antimicrobials tested of quinolones classes were only nalidixic acid and ciprofloxacin, where only resistance to nalidixic acid was observed but no resistance to ciprofloxacin. This discrepancy can be explained because of the mutations necessary to develop with a single resistance to each of these antimicrobials.

Although nalidixic acid and ciprofloxacin belong to quinolones class, resistance to the first antimicrobial is developed with a single chromosomal point mutations, whereas resistance to ciprofloxacin generally requires at least two chromosomal mutations (Crump et al., 2003). Quinolones are often a first-choice to treat *Salmonella* infections; ciprofloxacin exclusively is the first option to treat salmonellosis in adults (Mølbak, 2005). Although resistance to nalidixic happens without predisposing this same isolates to ciprofloxacin resistance, this still represents a risk to human health. A study conducted in Denmark observed that ciprofloxacin therapies to treat infections with *Salmonella* resistant to nalidixic acid but susceptible to ciprofloxacin were less effective compared with treatment of *Salmonella* susceptible to nalidixic acid. In this same study, it was observed that people infected with strains resistant to nalidixic acid were twice as likely to die than those infected with strains resistant to tetracycline, chloramphenicol, streptomycin, ampicillin but no resistance to nalidixic acid (Helms et al., 2002).

Aminoglycosides are used in the poultry industry. Here we measured resistance to gentamycin and streptomycin. While resistance to gentamicin was not observed, the presence of resistance to streptomycin was seen and can be the result of use this antimicrobial agent against necrotic enteritis, fowl cholera or *Staphylococcus* spp. (Landoni and Albarellos, 2015). While resistance to chloramphenicol was low, the use of this agent can increase in human medicine when access to cephalosporins or cepems is limited (Collinson et al., 2009). Ampicillin and combinations such as amoxicillin with clavulanic acid, are used to treat *E. coli* infections, but its use is not as common as penicillin in the poultry industry. Ceftriaxone, as well as ceftiofur, is an agent from the

cephems class which is used in poultry, however, including ceftiofur which is used in this industry for colibacillosis treatment (Landoni, and Albarellos, 2015).

Tetracycline, nalidixic acid, chloramphenicol, azithromycin, amoxicillin/clavulanic acid and ceftriaxone are antimicrobial agents that while their classes are of common use in the poultry industry, these agents are not (Hofacre et al., 2013; Landoni and Albarellos, 2015). There was no specific association between antimicrobial resistance patterns and *Salmonella* serotypes. The same finding was observed in Álvarez-Fernández et al. (2012) where there was no association between antimicrobial resistance and serotypes.

3.5 Limitations

Several limitations were found during the course of this study, specifically, the sampling frequency, and the sample types collected. Because this study was designed with commercial producers, our scheduling was based on the times provided by the producers and also, the access provided by the producers to the information on biosecurity practices that were followed. This study utilized isolates obtain from a previous study, therefore, there was no interaction with the farmers on our part to understand if there were any specific inconsistencies in implementing biosecurity practices. Another limitation for this study was the fact that we sampled in 2 different seasons that could add to the confounding factors for variable results such as different employees working in the farms, different animals, and seasonality.

Changes on prevalence of *Salmonella* were expected after improvement of biosecurity practices and management. Recommendations were made, but changes in practices were not adequately documented by the producers and farm staff, leading to inconclusive interpretations of the data collected. However, this does raise another concern with regards to appropriate training and education of employees to understand implications of improper biosecurity practices on the farms. Recommendations based on biosecurity practices and management of the farms were indirectly made to the farmers via the integrator, therefore, it is possible that there could have been some miscommunication. Because recommendations were made to the integrator, all the four farms received the same recommendations. The recommendations on improvement were not specific for each farm and their actual practices at that time. Regarding materials limitations, antimicrobial resistance was determined using the NARMS panel, which contain a total of fourteen different antimicrobial agents. Therefore, resistance to other antimicrobials of veterinary and medical importance were not tested.

Methodology for *Salmonella* identification was limited to serotyping which can not give any further information at strain level which could have been obtained through sequencing. Because the most common serotypes found in poultry are *S. Enteritidis* and *S. Typhimurium*, there is limited literature about *S. Berta*, *S. Mbandaka*, *S. Tennessee*, and *S. Kentucky*, thus limiting comprehensive assessment of the implication of these results.

CHAPTER 4. CONCLUSIONS AND FUTURE WORK

In this study, the prevalence of *Salmonella* in four different poultry farms during two seasons as well as their antimicrobial resistance patterns were determined. Values of *Salmonella* prevalence during the pre-recommendations season ranged between 3% and 4% and for the post-recommendations season 6% and 14% were the lowest and higher values respectively. Based on these results, it can be inferred that recommendations on the management and biosecurity practices had no effect on the prevalence values of *Salmonella*. In contrast, an increase in the *Salmonella* prevalence was observed for the post-recommendations seasons. However, these differences are not statistical significant.

This increase in *Salmonella* during post-recommendations season (October-November) compared to the prevalence during pre-recommendations season (March-April) can be attributed to seasonality, since previous studies have shown that *Salmonella* prevalence is higher during summer and fall than spring. Antimicrobial resistance was observed in 7% (19/262) *Salmonella* isolates.

Resistance to first choice antimicrobials to treat salmonellosis was not observed. However, resistance to clinically important antimicrobials such as ceftriaxone, nalidixic acid and azithromycin was exhibited by *Salmonella* isolates. Antimicrobial resistance was found to be different in each farm and between farms.

A total of six different serotypes were identified in this study including *S. Enteritidis* the most prevalence serotype, followed by *S. Berta*, and *S. Mbandanka* which were present in all four farms. *S. Typhimurium*, *S. Tennessee*, and *S. Kentucky* were only present on a single farm. All farms followed the same trend of having *S. Enteritidis* as the only serotype present during pre-recommendations season, and the presence of new serotypes during the post-recommendations season was observed. This study contributes to AMR studies and presence of AMR foodborne pathogens during pre-harvest poultry. Further studies such DNA fingerprinting could be carried out to determine if the AMR isolates are the same strain, and to determine if there is cross-contamination between these four farms. Additional studies could be performed to determine the source of AMR genes and how they are being transferred between bacteria. Presenting these results to farmers and the integrator could better inform biosecurity practices.

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APPENDICES

APPENDIX A

Table 7. Presumptive positive *Salmonella* in samples obtained from four poultry farms in two seasons.

Farm	Pre-Recommendations		Post-Recommendations		Total	
	No. of samples	Positive samples	No. of samples	Positive samples	No. of samples	Positive samples
1	240	19 (8%)	240	36 (15%)	480	55 (11%)
2	240	11 (5%)	240	32 (13%)	480	43 (9%)
3	240	14 (6%)	240	16 (7%)	480	30 (6%)
4	240	35 (15%)	240	35 (15%)	480	70 (15%)

(Information derived from English, 2015 thesis)

APPENDIX B

Table 8. Confirmed *Salmonella* in samples obtained from all four farms in both seasons.

Farm	Pre-Recommendations		Post-Recommendations	
	No. of samples	Positive Isolates	No. of samples	Positive Isolates
1	240	7 (3%) ^A	240	15 (6%) ^J
2	240	6 (3%) ^A	240	14 (6%) ^J
3	240	9 (4%) ^A	240	12 (5%) ^A
4	240	6 (3%) ^A	240	34 (14%) ^A

^A: Same letter represent no significant differences between season (p>0.05).