A MATHEMATICAL APPROACH TO UNDERSTAND FOODBORNE PATHOGENS IN PASTURED POULTRY FARMS IN THE SOUTHEASTERN U.S.

by

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(Under the Direction of Abhinav Mishra)

ABSTRACT

Foodborne pathogen contamination can occur at many points along the broiler supply chain. As a fast-growing alternative to conventional broiler production system, the pastured poultry system allows chickens to graze freely on pasture with a movable pen, in which more stringent rearing practices are applied. As a result, food safety concerns may differ from those found in conventional production. Currently, the food safety risks associated with alternative production strategies are little understood. The goal of the current study was to fill in information gaps about food safety risks present in alternative broiler production system. Preharvest (feces and soil) and postharvest (ceca and whole carcass rinse) broiler samples were collected from pastured poultry farms and processing plants in the southeastern United States. Machine learning models, such as random forest, least absolute shrinkage and selection operator (LASSO), and regression tree (RT) were constructed to predict *Campylobacter* prevalence and *E. coli* concentration in pre- and postharvest samples based on farm management practices. The RMSE (in $\log_{10}$ scale) under LASSO was 0.974 and 1.437, while under RT it was 1.032 and 1.476 for feces and soil samples, respectively. It was found that the source of animal feces was the important factor in predicting *Campylobacter* prevalence and *E. coli* concentration in feces and soil samples. Furthermore, logistic regression, a statistical model, was used to predict presence and absence of foodborne pathogens by the indicator microorganism generic *E. coli*. Additionally, the association between pastured-related microbiome and *Campylobacter* and *Salmonella* presence was investigated. Samples were assessed for *Campylobacter* and *Salmonella* using selective media and molecularly using microbiome relative abundances via 16S rRNA amplicon sequencing. In addition, Linear discriminant analysis (LDA) effect size (LEfSe) was used to identify taxa significantly enriched in *Campylobacter* and *Salmonella* positive samples or negative samples. Finally, a retail-to-consumption risk assessment model was used to assess seasonal effects of the risk of having contaminated broiler meat produced and prepared in-home in the United States. The model showed that higher number of infections and illnesses were estimated in the summer and fall months. These findings will aid in the broiler industry's risk-based decision making.

INDEX WORDS: pastured poultry; *Campylobacter*; *Salmonella*; *E. coli*; machine learning models; risk assessment

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DEDICATION

I would like to dedicate this work to my mother and father, and my boyfriend, An Chen. None of this work would be possible without your continued love, patience, and support. Thank you.

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CHAPTER 1

INTRODUCTION

1.1 Concerns about microbial contamination in poultry

Salmonella spp. and *Campylobacter* spp. are the top two foodborne pathogens causing millions of cases of infections in the United States every year (Thames and Sukumaran, 2020). Contaminated poultry meat is one of the leading causes of salmonellosis, with some studies claiming that poultry is responsible for 25% of outbreaks caused by foodborne pathogens (Akil and Ahmad, 2019). Chickens are also estimated to be responsible for up to 30% of human campylobacteriosis cases (Dogan et al., 2019). Horizontal transmission through old litter, untreated drinking water, domestic pets, other farm animals, wildlife, insects, contaminated feed, bird-to-bird pathogen transfer, farm equipment and vehicles, and farm workers are the main sources of *Campylobacter* and *Salmonella* colonization in chickens (Frosth et al., 2020; Heyndrickx et al., 2002; Sahin et al., 2002). During processing, head pulling and evisceration, where crop leakage and intestinal rupture are major sources of foodborne pathogen contamination (Smith et al., 2007). Moreover, scalding and defeathering are also identified as evident sources of contamination (Finstad et al., 2012). Meanwhile, cross-contamination is a major risk factor during processing and preparation (Rasschaert et al., 2008).

1.2 Pastured poultry production

Pastured poultry farms in the United States either allow birds to roam completely free on pasture or raise them in small, open-air moveable pens where birds have daily access to fresh pasture (Rothrock et al., 2019; Rothrock et al., 2016). In recent decades, there has been an increasing demand for free-range or pastured poultry products (Elkhoraibi et al., 2017). Surveys have shown that consumers are supportive of all-natural products (Dailey et al., 2017). Most pastured poultry producers are small family farms, which the USDA defines as farms that sell $100,000 in agricultural products per year (USDA/ERS, 2011). Because no clear food safety guidance is available for small farms and birds are exposed to a less controlled environment, the microbial risk of the chickens is increased. Food-borne pathogens need to be studied in pastured poultry farms to find out how common they are and what risks are linked to their presence.

1.3 Project objectives

The goal of this dissertation was to utilize statistical models to understand the prevalence of *Campylobacter* and its associated risk factors; possibility of using *E. coli* as an indicator organism; the connection between pastured-related microbiome and pathogen presence; and the risk assessment of the seasonal effect of *Campylobacter* from retail to consumption. Specifically, objectives were:

1. **To predict generic *E. coli* concentrations and *Campylobacter* prevalence in pastured poultry farms using farm management practices (Chapters 3 and 4).** Relatively little is known about what farm management factors are associated with *E. coli* concentration and *Campylobacter* presence. In these chapters, least absolute shrinkage and selection operator (LASSO), regression tree (RT), and random forest (RF) were used to figure out which farm variables are most important for predicting concentrations of *E. coli* and the presence of *Campylobacter*.
2. **To predict foodborne pathogens in pastured poultry farms by indicator bacteria (generic *E. coli*) (Chapter 5).** Information on the prevalence of foodborne pathogens and their correlation with *E. coli* in pastured poultry farms is limited. This chapter used

logistic regression models to show correlations and make predictions about foodborne pathogens.

3. **To compare differentiating taxa between cultural and microbiome positive and negative groups for *Campylobacter* and *Salmonella* (Chapters 6 and 7).** Little is known about the connection between pasture-related microbiomes and foodborne pathogens. There was a need to understand the different results obtained from cultural and microbiome methods. As such, the purpose of this chapter was to address the need.
4. **To present a quantitative microbial risk assessment (QMRA) model to estimate the seasonal trend of risk of campylobacteriosis by consuming broiler meat prepared at home in the United States (Chapter 8).** We identified that there were no QMRA models discussing the seasonal trend of risks associated with campylobacteriosis. This chapter shows a QMRA model that estimates the number of cases of campylobacteriosis each season in the United States that are caused by chicken cooked at home.

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

The United States has the largest broiler chicken industry in the world, producing over 59.75 billion pounds of broiler chicken in 2020 (NCC, 2020). Americans consume more than 98 pounds per capita in 2020, which is more than anyone else in the world (NCC, 2020). Food safety remains a key concern in commercial poultry production in this thriving industry. Effectively controlling and reducing food safety risks presents significant challenges for several reasons. Multiple steps from breeder flocks and hatcheries to commercial layer and broiler flocks make the production management complicated. In each of the steps, different environments in housing, feed management, transportation, and storage can cause a variety of food safety risks (Ricke, 2021). In addition, poultry processing safety is influenced by the status of the live broilers entering the processing plant. Foodborne pathogens can be brought into processing plants and then cause cross-contamination within the plant. The use of microbiome data to investigate the relationships between foodborne pathogens and other bacterial taxa is promising.

The microbiome is the collection of microorganisms that live symbiotically on and in the human or animal body, including the digestive system (Javitt and Javitt, 2018). It is made up of various bacteria, archaea, and eukarya. Microbiome is responsible for a variety of digestive system activities, including destruction of plant fibers, production of digestive chemicals, development of the immune system, and exclusion of pathogens. The chicken gastrointestinal tract (GIT) has a rich microbial population with more than 500 phylotypes or around one million bacterial genes (Sergeant et al., 2014; Wei et al., 2013b), which is 40 to 50 times the number of genes in the chicken genome. Unfortunately, traditional microbiological culture methods are unable to recover the majority of these microorganisms (Rappe and Giovannoni, 2003), with just 20% of the GIT bacterial species recovered (Gaskins et al., 2002). To detect these non-cultivable microbes, genome sequencing methods (16S rRNA amplicon sequencing, metagenomic sequencing, etc.) are required. Many of these microorganisms serve a significant function in the GIT of chicken, such as in the digestion of nutrients from feed (Kogut, 2019). Wei et al. (2013b) provided a summary of the chicken GIT microbiome's features. A total of 915 operational taxonomic units (OTUs) were found and categorized into 13 phyla, with the top three being *Firmicutes* (70%), *Bacteroidetes* (12.3%), and *Proteobacteria* (9%). The majority of *Firmicutes* are spore-forming, gram-positive, and very resistant to environmental stress (Parkes and Sass, 2009). Major families from *Firmicutes* include *Lachnospiraceae*, *Ruminococcaceae, Lactobacillaceae, Veillonellaceae*, and *Erysipelotrichaceae* (Rychlik, 2020). *Desulfohalobium, Escherichia/Shigella*, and *Neissenia* were the most prevalent genera within the phylum *Proteobacteria* (Wei et al., 2013b).

In addition to establishing the composition and ecology of the chicken GIT microbiome, several research (Crippen et al., 2019; Li et al., 2020; Luiken et al., 2020; O'Brien et al., 2016; Rothrock et al., 2016; Rothrock and Locatelli, 2019; Yang et al., 2019) have examined the microbiome of soil and dust in poultry farms, broiler carcass rinses during processing, and retail chicken meat products. Microbiome compositional features have been used to identify not just the impact of diet, age, breed, sample location, and feed additives, but also the link between foodborne pathogens in different sample types. The rising number of research concentrating on microbiome data acquired from farms, processing facilities, and retail establishments gives us the ability to comprehend, forecast, and ultimately minimize food safety risk. For instance, Park et al. (2017) showed *Lactobacillus* intestinalis to be a population-level predictor of *Campylobacter*. Liu et al. (2018) discovered that *S*. enteritidis injection considerably altered the microbial population at various time periods. After *S*. enteritidis inoculation, the research also revealed positive relationships between *Coprococcus* and *Flavonifractor* and *Bacillus* and *Blautia*. In the meanwhile, machine learning techniques such as Random Forest, Support Vector Machine, and Log-boost have been used to microbiome research, enabling the prediction of diseases based on microbiome data. This article examines the application and modification of microbiomes depending on sample type, including cecal, fecal, soil, dust, and whole carcass rinse.

2.2 The chicken gastrointestinal tract (GIT) and cecal microbiome

The chicken GIT consists of the following: crop, proventriculus, gizzard, small intestine, large intestine, ceca, and cloaca. The crop functions as a temporary food storage where the fermentation of lactate and breakdown of starch take place. Digestion starts at the proventriculus, where food is soaked in hydrochloric acid and digestive enzymes begin breaking down nutrients and forming the food bolus. The gizzard grinds and mixes the food bolus with gastric juices from the proventriculus and other organs. From the gizzard, food enters the small intestine, which is divided into three parts: the duodenum, jejunum, and ileum. Proteins are digested through aminopeptidase, amylase, maltase, and invertase and other nutrients are absorbed in the small intestine. The water and foods that are not digestible are passed to the large intestine where further absorption occurs. The ceca are a pair of tubes where undigested foods are fermented and are emptied every 24 h. Then the waste leaves the chicken GIT through the cloaca. Because of the huge variation and the high diversity of microbiomes between each section, it has been suggested that each section should be analyzed as a separate ecosystem (van der Wielen et al., 2002). As a result, we will discuss the characteristics of the microbiome based on sections. Specifically, we will focus on the cecal and fecal microbiomes, which are two of the most targeted GIT sections for food safety-related research and represent the most unique microenvironment along the GIT.

2.2.1 Composition of cecal microbiome

Many studies have investigated the composition and structure of the cecal microbiome where dominant taxa and unclassified species were identified. Wei et al. (2013b) identified *Firmicutes* and *Bacteroidetes*, accounting for approximately 78 and 11% of the total cecal sequences, respectively. Conversely, the bacterial community of Tibetan chicken consisted of *Bacteroidetes* (>47%), *Firmicutes* (>18.8%), and *Spirochaetae* (>0.3%) at phylum level (Zhou et al., 2016). Sergeant et al. (2014) discovered 699 phylotypes, more than half of which appear to be unknown species, whereas Pandit et al. (2018) discovered *Bacteroides* as the dominant genus, with unclassified *Clostridiales*, *Clostridium*, *Alistipes*, *Faecalibacterium*, *Eubacterium*, and *Blautia* as minor players. Similarly, Oakley and Kogut (2016) found *Bacteroides* significantly abundant in week one post-hatch chickens and that *Clostridium* and *Caloramator* significantly increased in week six for post-hatch chickens. Stanley et al. (2013) found large variations in the cecal microbiome within a single trial. The author postulated that the variability might be due to a lack of colonization of maternally derived bacteria. In addition, cecal microbiome composition is thought to change over time, breed, diet, and additives (De Cesare et al., 2019b; Diaz Carrasco et al., 2018; Feye et al., 2019; Ijaz et al., 2018; Van Goor et al., 2020; Yausheva et al., 2018).

2.2.2 Effect of pathogen colonization on cecal microbiomes

For decades, detection and identification of foodborne pathogens have depended on culture-based approaches. In the meanwhile, the connection between infections, food safety, and human health has been thoroughly investigated. As a rapidly expanding molecular-based approach, high throughput sequencing has been extensively used in epidemic investigation, surveillance, and determining microbial community features. This method allows for the investigation of interactions between diseases and commensal microorganisms and the environment. Intestinal infections by pathogens alter the diversity and relative abundance of bacterial species. In turn, a healthy microbiome in the gut offers protection against infections. Consequently, it is necessary to evaluate the effect of the complete microbial population on the presence or absence of pathogens. The features of microbiome data following pathogen infection in broiler chickens are summarized in Table 2.1.

2.2.2.1 Bacterial pathogens

Salmonella is a significant source of the biological threats posed by chicken. From 1998 to 2012, chicken was the most often implicated food in *Salmonella* outbreaks (Chai et al., 2017). Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture (USDA) stated that 22.2% of half-chicken carcasses and 24.0% of raw chicken parts in the United States tested positive for *S. enterica* (Li et al., 2020). *Salmonella* primarily colonizes the ceca of the GIT, which are dominated by *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* (Khan, 2014; Qu et al., 2008; Wei et al., 2013b). Liu et al. (2018) studied the alterations in the cecal microbiota after a challenge with *Salmonella* enteritidis in broilers. After *S*. enteritidis infection, a substantial shift in *Bifidobacterium* was seen. In addition, after 28 days of infection, a rising relative abundance of *Bacillus* was detected in chicken. *Salmonella* is able to use *Bacillus*-produced organic acids as a source of energy, which explains the favorable association between *Salmonella* infection and *Bacillus* growth (Bedford et al., 2000). Mon et al. (2015) examined the young layer chicken and discovered that *S*. enteritidis infection may dramatically reduce the total diversity of the microbiome in the stomach. Kempf et al. (2020) came to the conclusion that alterations in the microbiome of the chicken intestine may drastically change the outcome of *Salmonella* infection. After *Salmonella* infection, all the birds without a microbiome injection from adult chickens became super-shedders. Inoculation of the gut microbiota, on the other hand, protected chickens against *Salmonella* infection.

Campylobacter jejuni is the most common agent linked with such infections. One of the leading causes of *Campylobacter* infections is the improper handling or eating of raw, infected chicken meat (Luber et al., 2006). *Campylobacter* has been found as a significant reservoir in chicken due to its capability to colonize the gastrointestinal tract of chickens to the extent of 10^6 CFU/g in ceca (Rosenquist et al., 2003). In addition, the gut microbiota plays a crucial function in nutrition, the immune system, growth performance, and pathogen defense. However, little is known about how *C. jenuni* influences the microbiome in the chicken stomach. Ijaz et al. (2018) conducted a daily sample research with duplicates to examine the microbiome of the chicken intestine over time. Between days 12 and 20, the author suggests there may be a chance for *Campylobacter* colonization. Thibodeau et al. (2015) infected 14-day-old birds with *C. jenuni* and observed no significant change in alpha-diversity but a considerable shift in beta-diversity. When *C. jenuni* invaded the chicken ceca, the relative abundance of the taxa *Faecalibacterium* and *Clostridium* rose. In addition, the research postulated a connection between *C. jenuni* and *Clostridium* in which *C. jenuni* might serve as a hydrogen sink, so promoting the development of *Clostridium* through greater fermentation and resulting in increased organic acid synthesis. *C. jenuni* and chicken might then use the organic acid generated by *Clostridium* as a source of energy. A similar correlation was discovered by Awad et al. (2016), who administered *C. jenuni* orally into 14-day-old chicks and analyzed their jejunum and ceca microbiomes. The investigation revealed that the infected group had a greater relative abundance of *Clostridium* than the control group. It is hypothesized that *Campylobacter* infection increases intestinal mucus production (Molnar et al., 2015). Therefore, the increase in mucin secretion allows *Clostridium* species to grow (M'Sadeq et al., 2015). The greater prevalence of *Campylobacter* and *Clostridium* spp. may result in increased endotoxin generation and increased intestinal permeability, which favors colonization (Awad et al., 2016). The author also noted that *C. jenuni* dramatically decreased *E. coli* in the jejunum and ceca of infected broilers. This was also consistent with their earlier findings that *Campylobacter* colonization lowered *E. coli* counts in the jejunum and ceca at 7- and 14-days post-inoculation. These data imply that *E. coli* might be used as a significant indicator of *Campylobacter* infection in hens. A favorable tendency was seen for the *Alcaligenaceae* family with *Campylobacter* infection (Hankel et al., 2019), however it was not statistically significant. According to their findings, the family *Alcaligenaceae* was composed entirely of the genus *Parasuterella*. This genus has been identified as a fundamental component of both the human and mouse gut microbiome. It relies on amino acids such asparagine, serine, and aspartate to create succinate as a fermentation byproduct (Ju et al., 2019). *C. jejuni* consumes comparable amino acids as essential energy sources, hence it is predicted that a growth situation that favors *C. jejuni* should likewise benefit *Parasutterella*. Because both metabolisms depend on comparable amino acids, this is the case. In addition, a study (Patuzzi et al., 2021) examined the interaction between *Campylobacter* colonization and the cecal microbiota over time. *Bacteroidales* and *Clostridiales* were substantially more prevalent in persons from *Campylobacter*-positive farms than in those from farms that remained negative.

Examining the relationships between bacterial taxa and *Campylobacter* Ocejo et al. (2019) found a negative link between *Campylobacter* and the species *Sutterella* (order

Burkholderiales), which has been identified as *Campylobacter*'s direct competitor in the gut environment. Another species adversely connected with *Campylobacter* was *Parabacteroides* (phylum *Bacteroidetes*), which has been linked to a healthy gut microbiome because of its potential to promote host immunity (Kverka et al., 2011). Contrary to *Lactobacillus*, *Parabacteroides* correlated strongly with *Campylobacter* (Ocejo et al., 2019). In addition, Park et al. (2017) discovered that *Lactobacillus* intestinalis was a predictor of *Campylobacter* population numbers in fructooligosaccharide-treated mice versus controls.

Another infection that leads to necrotic enteritis and economic loss is *Clostridium perfringens*. Kiu et al. (2019) discovered that the cecal microbiomes of *Clostridium perfringens*-infected chickens did not vary substantially from those of healthy chickens, despite a favorable correlation with the genus *Clostridium*. A study examined the impact of *Bacillus licheniformis* supplementation on chickens challenged with *C. perfringens* and *Eimeria*. The findings revealed that the structure of the cecal microbiome was identical to that of the control groups, indicating that the probiotic had a diminished impact on the pathogen (Lin et al., 2017).

Eimeria spp. are pervasive infections that have a significant impact on chicken production, producing mucosal injury and dysbacteriosis and thereby impacting the health of the microbiome in the gut (Dalloul and Lillehoj, 2006). Macdonald et al. (2017) discovered that *Eimeria tenella* infection dramatically altered the cecal microbiota at the order and genus levels. Even in asymptomatic chicks, *Bacteroides* decreased significantly while *Lactobacillus* increased. After *Eimeria* infection, the cecal microbiome was dominated by *Escherichia/Shigella* and *Bacteroides* at the genus level, according to a recent research (Martynova-Van Kley et al., 2012). However, Eimeria infection had no significant effect on the diversity of the microbial community in the cecum.

2.2.2.2 Virus Pathogens

Avian leukosis virus (ALV), a member of the retroviridae family, is a pathogen in chickens and has been documented to create gut microbiome disturbance, which may promote viral growth and transmission. Ma et al. (2017) discovered that ALV-infected birds favored opportunistic pathogens such as *Staphylococcus* and *Weissella* in Firmicutes and certain genera in *Bacillales*. The findings suggested that the cecal microbiome may distinguish between healthy and sick chickens.

2.2.3 Effect of flock management on cecal microbiomes

2.2.3.1 Conventional and cage free

Van Goor et al. (2020) discovered that the cecal and lung microbiomes of both conventional and cage-free chickens had three main phyla: Bacteroidetes, Firmicutes, and Proteobacteria. Furthermore, the cecal microbiota of slow-growing free-range birds was more diverse and complicated than that of conventional chickens (Ocejo et al., 2019).

2.2.4 Effect of animal behavior on cecal microbiome

Changes in the composition of the gut microbiome are associated with illnesses in humans, including hyperactivity disorders, depressive disorders, and autistic spectrum disorders (Dash et al., 2015; Jia et al., 2008; Mulle et al., 2013). Multiple studies have shown the connection between the gut microbiota and the behavior of chickens. A study comparing the cecal microbiomes of high feather-pecking (HFP) and low feather-pecking (LFP) chickens revealed that the LFP birds had much greater beta diversity (Birkl et al., 2018). HFP chickens had a higher abundance of *Clostridiale* and a lower abundance of *Lactobacillacae* compared to LFP chickens.

2.3. Poultry fecal microbiomes

2.3.1 Composition of fecal microbiome

Several studies have examined the structure and composition of the chicken fecal microbiome. Hieke et al. (2019) discovered that the fecal microbiome did not accurately reflect the cecal microbiota. Collecting fecal and cecal samples from laying hens during their first 19 days of life revealed that both included *Firmicutes* and *Bacteroidetes* at the phylum level, but with a clear distinction at the family level. Cecal samples were dominated by *Ruminococcaceae* and *Lachnospiraceae*, and fecal samples were dominated by *Lactobacillaceae*, *Ruminococcaceae*, *Clostridiaceae 1*, and *Lachnospiraceae* (Hieke et al., 2019). Similarly, Oakley and Kogut (2016) investigated the fecal and cecal microbiomes at various stages of chicks and found that *Lactobacillus* and *Gallibacterium* were considerably over-represented in fecal samples at week one post-hatching. Cecal samples had more *Bacteroides*, *Pseudoflavonifractor*, *Oscillibacter*, *Flavonifractor*, and *Subdoligranulum* than fecal samples. Moreover, according to a study, while 88.55% of all OTUs (including 99.25 percent of sequences) were shared across fecal and cecal samples of broilers, the bacterial community structure changed considerably according to either alpha or beta diversity (Stanley et al., 2015). This result is consistent with the findings of Oakley and Kogut (2016) and Hieke et al. (2019). In addition, Sekelja et al. (2012) investigated the relationship between the chicken fecal microbiome and its genesis in the gastrointestinal tract. There were four main phylogroups identified in the fecal microbiome. Two *clostridial* phylogroups were associated with the microbiome of the cecum and small intestine, whereas the *lactobacillus* group was associated with the microbiome of the upper gut (crop and gizzard). Despite the possibility that the fecal microbiome is not typical of cecal samples, there is a link between the fecal microbiome and other gastrointestinal regions. Yan et al. (2017) revealed that the microbiome composition of the duodenum, ceca, and feces considerably varied. According to the examination of similarities, the duodenum microbial community was more comparable to the fecal than the cecal community. The Shannon index of ceca was favorably connected with *Firmicutes,* negatively correlated with *Bacteroidetes,* and weakly correlated with feces. This provides more evidence that the fecal microbiota differs greatly from the cecal microbiome. Vidенska et al. (2014) discovered that *Firmicutes* (76.2%), *Proteobacteria* (14%), and *Bacteroidetes* (6.5%) dominated the fecal bacterial population. *Lactobacillus* was the most prevalent taxon at the genus level, followed by *Peptostreptococcaceae*, *Streptococcus*, and *Escherichia*. In addition, the families *Clostridiaceae, Ruminococcaceae*, *Lachnospiraceae*, and *Veillonellaceae*, as well as the classes *Bacteroidia* and *Actinobacteria*, were prevalent in the fecal microbiome.

2.3.2 Effect of pathogen colonization on fecal microbiomes

2.3.2.1 Bacteria pathogen

Researchers examined the association between *Campylobacter concisus* and the fecal microbiota of 56-day-old broiler chickens (Kaakoush et al., 2014). Compared to *C. concisus*-negative samples, the presence of *C. concisus* was related with a reduced abundance of *Lactobacillus* and *Corynebacterium* and a greater abundance of *Streptococcus* and *Ruminococcus*. This finding demonstrated the possibility for predicting the presence of *Campylobacter* using fecal microbiomes. In contrast, another research that collected fecal samples in a commercial broiler chicken production facility found no significant connection between *Campylobacter* and other taxa at the species level (Oakley et al., 2013). This finding gave an explanation for the ineffectiveness of competitive exclusion tactics against *Campylobacter*. The dearth of co-occurring species that are expected to fill the biological niche space occupied by *Campylobacter* is the primary cause of its inefficiency. Dicksved et al. (2014) studied the fecal microbiome makeup of spontaneously infected chickens with *Campylobacter* versus uninfected chicks. The proportions of *Bacteroides* and *Escherichia* in the *Campylobacter* positive group were substantially greater than in the *Campylobacter* negative group. This result was in line with the finding of Awad et al. (2016) consistent with Awad et al. (2016) conclusion that *Campylobacter* colonization decreased the *E. coli* burden in the jejunum and ceca. The chickens subsequently excrete *E. coli* in their feces, hence boosting the *E. coli* population in fecal samples. In addition, greater proportions of *Phascolarctobacterium* and *Streptococcus* were found in the *Campylobacter*-positive group compared to the *Campylobacter*-negative group, despite the fact that these taxa had not been previously related with colonization resistance (Dicksved et al., 2014). Kaakoush et al. (2014) showed that the presence of *C. concisus* was related with a lower abundance of *Lactobacillus* and *Corynebacterium* and a greater abundance of *Streptococcus* and *Ruminococcaceae* in the *Campylobacter* positive group compared to the *Campylobacter* negative group. In addition, *Campylobacter* colonization was associated with *Bacteroides*, *Alistipes*, and *Blautia*, which produce a substantial amount of short-chain fatty acids (SCFA). *C. jejuni* may utilize acetate and lactate as carbon sources.

2.3.2.2 Effect of flock management on fecal microbiomes

Schreuder et al. (2019) investigated the shift in fecal microbiota composition after oral injection with wild duck feces. On day two, the relative abundance of the genus *Alistipes* was much higher in the duck feces inoculation group than in the control group; however, on day seven, the relative abundance of *Alistipes* increased in the control group. This implies that the introduction of duck feces may create a transitory shift in the fecal microbiome, but does not affect the microbial population significantly. Using fecal microbiomes, two pastured poultry farms were evaluated in comparison (Rothrock and Locatelli, 2019). The fecal microbiota was shown to be positively linked with aluminum, iron, manganese, silicon, and zinc concentrations in the feces of hens from farm one, but not from farm two. In addition, taxonomic diversity and beta-diversity differed dramatically between the two farms. In laying hens, heat stress may also alter the fecal microbiota (Zhu et al., 2019). The top three dominating phyla are *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*. With heat stress, however, *Firmicutes* reduced dramatically while *Bacteroidetes* proliferated. In addition, the modifications of the fecal microbiota showed metabolic pathways that explain the effect of heat stress.

2.4. Other Pre-Harvest Poultry-Related Environmental Microbiomes

2.4.1 Poultry litter

The growth cycle for commercial broiler production is around 6 to 7 weeks. On day one of each cycle, young chicks are housed in chicken homes at a high density (0.1 m^2) with litter as bedding material (Wang et al., 2016). Poultry litter is a combination of bedding materials (e.g., wood shavings), rice hulls, chicken feces including GI bacteria, feed, uric acid, and other items derived from broilers (e.g., feathers). Multiple studies have indicated that chicken litter includes a dynamic and complex microbiome, and that the microbiome of poultry litter is dependent on the environment of poultry houses and litter management protocols (Crippen et al., 2021; Lovanh et al., 2007; Lu et al., 2003). The selection of various bedding materials may influence the structure and content of the litter microbiome (Torok et al., 2009). De Toledo et al. (2020) described the impact of adding acidifiers, alkalizers, adsorbents, agricultural gypsum, and superphosphate to litter. In the comprehensive review and meta-analysis, litter treatments were shown to reduce pathogenic microbiomes. The reuse of chicken litter between successive flocks is a widely established management strategy in broiler production and another element influencing the microbiome of the litter. In the United States, chicken litter is often utilized for six or more successive growth cycles prior to a full cleaning to decrease the expense of new litter (Coufal et al., 2006). The reuse of chicken litter results in chemical and microbiological changes in the litter, while improper litter management may lead to an increase in moisture content, ammonia, pH, and the number and variety of microorganisms. A study reported the application of probiotic-based cleaning agents on spent litter, demonstrating a favorable impact on microbial decontamination (De Cesare et al., 2019a). The intake of litter particles by young chicks makes litter a driving factor in molding the microbiome of the broiler's gastrointestinal tract (Cressman et al., 2010). In addition, the microbiome of chicken litter was believed to correlate with *Campylobacter* isolation (Valeris-Chacin et al., 2021), while Kassem et al. (2010) demonstrated that *Campylobactor jejuni* and *Campylobactor coli* may live longer in reused litter than in fresh litter. *Salmonella* (Roll et al., 2011) and *Clostridium perfringens* (Wei et al., 2013a) levels in re-used litter were also found to be lower.

2.4.2 Farm soil

A microbiome is a complex ecosystem that contains information about different types of microorganisms. The composition and variability of the soil microbial community were of particular interest due to their capacity to demonstrate the relationship between the surroundings of a chicken coop and the soil microbiome. Crippen et al. (2019) investigated the soil microbiota of a chicken coop. Initially, *Firmicutes* and *Bacteriodetes* dominated the soil at the phylum level, but the *Bacteriodetes* population decreased soon following the addition of bedding and livestock. Within one flock rotation, *Firmicutes* and *Actinobacteria* dominated the soil, and this community was maintained throughout future flock rotations and partial clean-outs. *Actinobacteria* were prevalent in soil, where *Streptomyces* spp. generated antibiotic-producing secondary metabolites.

Clostridia, of which many are harmful, inhabit soil and the gastrointestinal system. Crippen et al. (2019) discovered *Clostridium sensu stricto*, types IV and XI in the chicken coop. In the first soil samples, cluster XI was the densest. During flock rotations, it was also discovered that *Staphylococcus* was widespread. However, *Staphylococcus* was not detected in the original microbiome of soil. *Campylobacter* was detected in soil samples, however no colonization was reported following the introduction of bedding and hens.

Rothrock and Locatelli (2019) studied soil samples from two pastured poultry farms and discovered that the soil microbiome differed considerably between the two farms. In the research, two farms had identical diets, but their fecal microbiomes were distinct. The discrepancy may be attributable to soil consumption. On farm one, the association between soil nutrient concentrations and its microbiome was statistically significant, however on farm two, no such correlation was seen. In addition, for both farms, a greater proportion of soil OTUs were found to be shared only with processing chicken samples than with fecal samples. This demonstrates that the environment of pastured poultry farms, particularly the soil, has a lasting influence on chicken processing.

2.4.3 Farm dust/bioaerosols

Airborne infections may quickly spread across poultry farms and homes. Animal feces are a significant cause of dust in agricultural settings. As a possible route of transmission, Luiken et al. (2020) investigated the association between the dust microbiome and feces in European chicken farms. The number of *Clostridia* was greater in dust than in feces, although *Bacilli* predominated in both dust and feces. The association between the bacterial population in dust and the resistome was statistically significant (0.65, p 0.0001), demonstrating that the dust microbiome impacts the makeup of the resistome. In a separate study (O'Brien et al., 2016), several forms of dust, including inhalable litter sampling dust (LS), settling dust (S), and inhalable mortality collection (MC), were examined in chicken buildings. Bacteria comprised the majority (64–67%) of all three forms of dust, followed by viruses (25–29%). *Proteobacteria* (33%, 37%, and 35%), *Firmicutes* (28%, 27%, and 28%), and *Actinobacteria* (19%, 15%, and 16%) were the three most prevalent phyla in LS dust, settled dust, and MC dust, respectively. At the genus level, the settling dust was identified as *Enterococcus*, *Bacteroides*, *Vibrio*, and *Clostridium*. In contrast, LS dust is widespread in the *Actinobacteria* phylum, the *Brachybacterium* genus, and the *Dermabacteraceae* family. The Kruskal-Wallis test (p 0.05) revealed that the microbiota composition of settling dust and LS dust differed considerably.

2.5. Post-harvest poultry microbiomes

2.5.1 Processing samples

Controlling the food safety of chicken production is an integral aspect of the farm-to-table food supply chain. Multiple causes of contamination exist, including processing, GIT, equipment, and food workers. The microbiome profile of rinsing a complete chicken carcass is capable of describing the taxonomic shift that occurs throughout processing, hence showing a possible correlation with pathogen presence. At rehang, pre-chill, and post-chill sampling sites, complete chicken carcasses were collected (Handley et al., 2018). According to the microbiome data, proteobacteria comprised 48% of all taxa recovered, followed by *Firmicutes* (31.7%) and *Bacteroidetes* (11.7%). In addition, Shannon diversity values declined dramatically from the least processed stage (rehang) to the most processed steps (pre-chill and post-chill), showing that the washing step eliminated a portion of the bacterial community. Similar investigations of compositional changes throughout chicken production have been conducted. Kim et al. (2017) collected samples from several processing stages, such as the bleed-out tunnel, the picker, the evisceration, and the chiller before to and after processing. *Firmicutes* (69.24%), *Proteobacteria* (15.15%), *Bacteriodetes* (10.19%), *Actinobacteria* (3.17%), and *Cyanobacteria* (0.95%) accounted for 98.70% of the total phyla. Similar patterns were seen across sample locations, with somewhat different dominating phyla. Similar to what Handley et al. (2018) discovered, the variety of early processing groups was greater than that of later processing locations. In addition, Wages et al. (2019) investigated whole bird carcass rinse (WBCR) collected at the post-scalder and post-picker stages of production at three processing plants. The diversity of microorganisms was greater in samples obtained after the scalder than in ones collected after the picker. Plant C had the biggest variance between sample sites, whereas plant B had the least. Plant A's post-scalder microbiome was dominated by *Firmicutes, Proteobacteria,* and *Bacteroidetes,* whereas Plant B contained the same taxa but in a different proportion. Interestingly, no *Salmonella* nor *Campylobacter* OTUs were found. In their place, closely related taxa like as *Erwinia, Serratia*, and *Arcobacter* were identified. Recent research conducted in Australia gathered samples before and after scalding, before and after immersion chilling, and after air chilling (Chen et al., 2020). In the majority of sample groupings*, Firmicutes, Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* were the four most prevalent phyla. For *Firmicutes*, the population varied from 34.5% in the group exposed to air-chilling to 77.6% in the group exposed to immersion-chilling. *Bacteroidetes* was a relatively rare phylum, ranging from 0.2% in the group before scalding to 5.7% in the group after air-cooling. The microbial community varied across sample groups at the genus level. In addition, *Lactobacillus, Staphylococcus*, and unclassified *Lachnospiraceae*, which are often found in the skin or GIT of chickens, were detected throughout the processing line, suggesting cross-contamination between processing stages. In addition to chicken carcass rinse samples, Rothrock et al. (2016) collected scalder and cooling tank water samples for microbiota study throughout the day in a chicken processing facility. The bacterial richness of the scalder tank grew during the day, particularly carcass- and feces-related bacteria, but reduced throughout the course of the processing day.

2.5.2 Final Products/Retail meat

The second-to-last point of consumption for customers is poultry items sold at retail. Understanding the microbiome profile of retail chicken meat is crucial for gaining a comprehensive understanding of food safety regulation and potential pathogen connections. A recent study in Korea evaluated packaged chicken meat from four different firms and unpackaged raw chicken carcasses from four separate butcher shops in January (when slaughter rates are lowest) and July (when slaughter rates are highest) (Kim et al., 2019). In both packaged and unpackaged meats, the number of bacteria was greater, and the diversity index was lower in July samples than in January samples. According to their findings, high temperatures in July may boost the development of dominant bacteria while inhibiting the variety of the bacterial population in chicken flesh. The microbiome composition of January samples differed from that of July samples, with Proteobacteria decreasing and Firmicutes increasing. However, Li et al. (2020) reported no statistically significant variation in the Shannon index between January and July retail chicken breast samples. It is unknown if production volume, production system, or climate change influence the makeup of the microbiome. Kim et al. (2019) evaluated the microbiome composition of *S*. Virchow under washed and unwashed conditions at temperatures of 27 and 4 ℃ in a subsequent experiment in which *S*. Virchow was intentionally infected. The risk of foodborne disease may be lowest for chicken meats held at 4 ℃ after washing, whereas it may be greatest for poultry products stored at 27 ℃ after washing, based on the change in microbiota makeup.

Different from the viewpoint of food safety, Lee et al. (2019) investigated the microbiome features of retail chicken items with various labels, such as organic, free-range, and antibiotic-free. Comparing the beta diversity of microbiomes across labels revealed no statistically significant differences. Rather than distinct production processes (labels), the commonalities across microbiomes are mostly due to the components of poultry products and their manufacturers. Lee et al. (2019) evaluated the prevalence of pathogens in addition to the taxonomic analysis. The conventional PCR analysis for Salmonella and *Campylobacter* was negative for all samples, agreeing with the taxonomic analysis, which detected no Salmonella genus and a negligible quantity (less than 0.6%) of *Campylobacter* genus. This result is consistent with Rouger et al. (2018) conclusion that no *Salmonella* nor *Campylobacter* were detected in chicken legs kept at 4 °C.

A recent study shown that packaging style and processing environment, but not antibiotic use and seasonality, contributed to the composition and diversity of the microbiome of chicken breasts sold in retail outlets (Li et al., 2020). Both the alpha and beta diversity indices demonstrated a statistically significant difference between vacuum-packed and air-permeable items. *Aeromonas* (20.7%), *Buttiauxella* (11.0%), *Carnobacterium* (22.9%), *Enterobacter* (2.9%), *Hafnia* (8.4%), and *Lactococcus* (6.0%) were the most prevalent genera detected in vacuum-packaged items, but *Pseudomonas* (87.3%) dominated air-permeable products

2.6 Modeling microbiome data

2.6.1 General information about microbiome data

The sequencing of 16S rRNA begins with sample collection, DNA extraction, and library assembly. Sequences must be allocated using either phylotype or OTU techniques in order to create datasets appropriate for further statistical studies. The phylotype technique assigns sequences directly based on their closeness to the annotated sequences previously deposited in databases, using the best-matching sequence in the reference database. The OTU method classifies sequences based on their resemblance to operational taxonomical units, as determined by a distance matrix at a predetermined threshold. The Phylotype technique offers a number of advantageous characteristics, including the ability to easily connect a sequence to previously recognized microorganisms, stable categorization, and computing efficiency. However, the effectiveness of assignment is strongly dependent on the sequencing platform and reference database, such that the database's dependability has a substantial impact on the results (Tyler et al., 2014). Additionally, this characteristic makes it challenging to describe fresh sequences. The OTU technique, on the other hand, allocates sequences based on sequence similarity, therefore it does not need previous information from the reference database (Chen et al., 2013). The OTU approach may catch all sequences, even those that have not been annotated and new microorganisms that have not been cultivated, making it a helpful tool for analyzing communities of microbes that are poorly described. Although the OTU method overcomes the limitations of the phylotype method, it has several flaws, such as sequencing errors, the absence of a consistent threshold for defining OTUs due to heterogeneous evolution rates, and the inability to determine which method to use to cluster sequences into OTUs (Chen et al., 2013; Schloss and Westcott, 2011).

After taxonomy assignments, the final datasets consist of tables of read counts and relative abundance with samples in the columns and OTUs in the rows. Microbiome data contains numerous aspects. The OTU table counts are inherently limited, high-dimensional, sparse, and dominated by zeros. This may result in complicated covariance, correlation between OTUs, and overdispersion with substantial within-group heterogeneities. The characteristics of microbiomes provide a novel approach to characterize microbial populations, but they also pose obstacles for modeling microbiome data. Examples include 1) how to include phylogenetic tree information; 2) how to decrease compositional limitations and address high p and small n difficulties; 3) how to manage uncommon species; and 4) how to model microbiome data with over-dispersion and zero-inflation (Xia and Sun, 2017). There are conventional statistical tools, freshly created multivariate statistical methods, and machine learning algorithms for addressing various elements of research problems, despite the severe limits of microbiome data analysis.

2.6.2 Use of predictive models for cultural data in poultry production

To evaluate food safety hazards in chicken farms, production, and retail, mathematical techniques including predictive modeling, random forest, classification and regression trees, and gradient boosting machines have been used. These models allow food makers and researchers to determine the pathogen-influencing characteristics of various raising and processing processes. Figure 2.1 depicts the microbiota analysis process flow. Golden et al. (2019b) discovered that the expected likelihood of isolating *Listeria* from fecal and soil samples from pastured chicken farms was greatest during the colder days of the year. Similar results were obtained when the expected chance of finding *Listeria* in soil samples from pastured poultry farms declined from over 0.35 to near zero when the lowest temperature two days before to sampling was over 20 °C (Golden et al., 2019a). Using logistic regression and classification trees, Ivanek et al. (2009) also associated the chance of isolating *Listeria* to weather and soil parameters in the natural setting. Strawn et al. (2013) estimated the frequency of *Listeria* in fruit and vegetable fields; the occurrence of *Listeria* was associated with soil accessible water storage, temperature, and land cover types. In addition, Pang et al. (2017) showed that wind speed and precipitation impacted the prevalence of *Listeria*, suggesting that runoff and wind-driven dust may be possible transmission pathways in mixed produce and dairy farms. The incidence of *Salmonella* and *Campylobacter* was evaluated for farm management methods and climatic conditions (Hwang et al., 2020a; Hwang et al., 2020b; Xu et al., 2021), with the exception of *Listeria*. Using a random forest model, Hwang et al. (2020b) examined the most important parameters in isolating *Salmonella* from pastured poultry farms for fecal, soil, whole carcass rinse of processing (WCR-P), and whole carcass rinse of finished product (WCR-F). Longer years of farming were associated with a greater projected likelihood of isolating *Salmonella* in the fecal, soil, and WCR-F models. Xu et al. (2021) showed that the presence of *Campylobacter* was related with various kinds of fecal samples taken from pastured chicken farms, and that a soy-free diet reduced the likelihood of detecting *Campylobacter*. Modeling reveals the relationship between farm management techniques or climatic parameters and the incidence of pathogens in the agricultural environment.

Since the late 1990s, quantitative microbiological risk assessment (QMRA) has been extensively utilized in the food sector to quantify the hazard risk to food consumers. QMRA models assist assess the risk by combining numerous significant supply chain components, while predictive models often concentrate on a specific circumstance. Golden and Mishra (2021) examined the risk of salmonellosis from retail to consumption for conventionally and alternatively produced chicken meat. According to the author, the median risk of infection per serving was comparable, but the average number of yearly infection cases was 641,000 for alternative meat and 3,800,000 for traditional meat.

2.6.3 Applicability of using microbiome data for poultry production

In the food business, predictive models and QMRA are valuable techniques for assessing food safety hazards. Neither of the models in the present research, however, use microbiome data. Microbiomes, in contrast to conventional microbiology methods, give more comprehensive and thorough information on not just pathogen species but also other microorganisms. Changes in microbiome composition are associated with the presence of pathogens, such as *Salmonella* and *Campylobacter*. By incorporating such microbiome data into prediction or risk analysis algorithms, fresh risk inputs may be generated.

Under a range of situations and treatments, microbiome data has been utilized to define the microbial ecosystem of farms, processing facilities, and food producing animals. For instance, the gut microbiota of broilers was studied (Mulle et al., 2013; Stanley et al., 2013; Wei et al., 2013b), as well as the reaction to food, additives, probiotics, prebiotics, and infections (De Cesare et al., 2019b; De Cesare et al., 2020; Diaz Carrasco et al., 2018; Feye et al., 2019; Kaakoush et al., 2014; Kempf et al., 2020; Mon et al., 2015; Nelson et al., 2020; Yausheva et al., 2018; Zhu et al., 2020). In the farm-to-fork supply chain, the microbiome is also employed to understand the farm and processing plant environments. For instance, soil and dust samples were collected from both conventional and pastured poultry farms (Locatelli et al., 2017; Luiken et al., 2020; Rothrock and Locatelli, 2019). During the processing of the whole chicken carcass, samples of water and rinse water were analyzed (Chen et al., 2020; Handley et al., 2018; Kim et al., 2017; Rothrock et al., 2016).

Particularly intriguing is the possibility that microbiome data may be utilized to monitor and forecast foodborne diseases. Bolinger et al. (2021) studied chicken rinse samples from different processing steps in order to predict the presence of *Salmonella* using machine learning techniques. Random forest had the highest levels of precision (88%), sensitivity (85%), and specificity (90%). It established the viability of using high-throughput sequencing technologies with poultry microbiome data to analyze indicator taxa and predict the presence or absence of pathogens. Farmers, poultry producers, and retailers are able to avoid identifying specific bacteria using culture techniques and instead understand microbial community information for disease risk analysis and evaluation thanks to microbiome data. In addition, microbiome data may be utilized to discover possible suggesting taxa, which may indicate pathogen contamination. Keeping track of changes in the microbial population may aid food safety managers in identifying trends and monitoring their plants (Bolinger et al., 2021). For instance, the finding of taxa connected with a disease is an indication of possible contamination, which necessitates additional analysis of the pathogen in order to prevent contamination. However, there are constraints, such as the quantification of microorganisms, the difference between living and nonliving organisms, the biological significance of functional prediction, and sample bias and variance. This must be overcome to correctly anticipate the presence of pathogens and provide consistent findings.

2.7 Conclusions

The gastrointestinal microbiome has a crucial role in host health, physiological development, immune system function, nutrition, and productivity, while fecal, soil, dust, and entire carcass microbiomes reveal health status and safety problems. The manipulation of the gut microbiota by feed additives or diets improves growth and enables the creation of a cost-effective feeding regimen. In the meanwhile, knowledge of various microbiomes across the processing chain improves food safety management methods at farms, processing companies, and retailers. The microbiome provides a novel tool for illuminating the relationship between farm management, the environment, and the host. In addition, the chicken microbiome has the potential to be employed in risk assessment and prediction models, giving food regulators and the poultry sector with more specific information for decision-making.

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Table 2.1 Characteristics of microbiome data after pathogen infection in broilers

Refence	Age of chicken	Pathogen	Sample TYPE	Does pathogen infection significantly affect alpha-diversity?	Does pathogen infection significantly affect beta-diversity?	Dominant taxa after infection	Taxa correlated with the pathogen	Changes of taxa after pathogen infection
Liu et al. (2018)	16, 23, 30, and 37 days	*Salmonella* Enteritidis	Ceca	Yes		*Firmicutes*, *Bacteroidetes*		Positive correlation between *Bacillus* and *Blautia*; positive correlation between *Coprococcus* and *Flavonifractor*
Mon et al. (2015)	3 days	*Salmonella* Enteritidis	Ceca	No	No	*Actinobacteria*, *Proteobacteria*		A marked decrease in *Enterococcaceae*, *Clostridiaceae*, *Lachnospiraceae*, *Peptostreptococcaceae*, *Ruminococcaceae*, and *Erysipelotrichaceae* was found in the *S*. Enteritidis-infected group at age of 3
	8 days	*Salmonella* Enteritidis	Ceca	No	Yes	*Actinobacteria*, *Proteobacteria*		Higher abundance level of *Enterococcaceae*,

						Clostridiaceae, *Peptostreptococcaceae*, *Erysipelotrichaceae*, and *Enterobacteriaceae* were found in the *S.* Enteritidis-infected group than in the non-infected group at age of 8
Thibodeau et al. (2015)	35 days	*Campylobacter jenuni*	Ceca	No	Yes	A decrease of *Streptococcus*, unclassified *Clostridia*, *Blautia*, *Anaerofilum*, *Christensenellaceae*, *Coprobacillus*, *Tenericutes*, *Mollicutes*, *Anaeroplasma* was found after *C. jejuni* colonization; A increase of unclassified *Lachnospiraceae*, *Mogibacteriaceae*, *Faecalibacterium*, *Clostridiaceae*, *Clostridium* was observed after *C. jejuni* colonization.

Awad et al. (2016)	7, 14 days	*Campylobacter jenuni*	Ceca	Yes		*Firmicutes*	*Campylobacter* infection caused a significant decrease in *E. coli*.	The relative abundance of *Clostridium* spp. was higher in the infected birds compared with the negative controls.
Hankel et al. (2019)	21, 70 days	*Campylobacter jenuni*	Ceca	No		*Firmicutes* (91.1%) and *Proteobacteria* (5.02%).	In young chickens, there was a positive Spearman's rank correlation between absolute abundances of *Campylobacteraceae* and *Alcaligenaceae*; there was a negative Spearman's rank correation between *Campylobacteraceae* and *Comamonadaceae*,	

							Campylobacteraceae and *Erysipelotrichaceae*.	
Stanley et al. (2012)	23 days	*Clostridium perfringens*	Ceca	No	Yes	*Clostridiaceae* (54.7%), *Lactobacillaceae* (8.56%)		
Kiu et al. (2019)	19-25 days	*Clostridium perfringens*	Ceca	No	No	*Bifidobacterium*, *Lactobacillus*		
Macdonald et al. (2017)	26 days	*Eimeria tenella*	Ceca	No	No			Order *Enterobacteriaceae* were increased while *Bacillales* and *Lactobacillales* were decreased in *E. tenella* infected group compared to control group
Martynova-Van Kley et al. (2012)	26 days	*Eimeria* spp.	Ceca	No		Genera *Escherichia*/*Shigell*a (up to 61.5%) and *Bacteroides* (up to 48.8%).		
Zhou et al. (2017)	19 days	*Eimeria tenella*	Ceca	No	Yes	*Firmicute* (89.67%), *Proteobacteria* (8.91%)		*Bacteroides dorei*, *Ruminococcus albus*, and *Romboutsia sedimentorum* were significantly decreased in

							infected group compared to control group
Kaakoush et al. (2014)	56-day	*Campylobacter concisus*	Fecal			*Firmicute*	*Escherichia*, *Alistipes*, *Enterococcus*, *Bacteroides*, *Shigella*, *Gallibacterium*, *Campylobacter*, *Faecalibacterium*, *Blautia*, *Enterobacter*, and *Clostridium* were associated with *Campylobacter* infection. The presence of *C. jejuni* was also associated with a lower abundance of *Lactobacillus* and *Corynebacterium* and a higher abundance of both *Streptococcus* and *Ruminococcaceae*.

Dicksved et al. (2014)	*Campylobacter* spp.	Fecal	No	Genus *Bacteroides* and *Escherichia*	The abundance of *Bacteroides* sequences was higher in *Campylobacter* positive group than in *Campylobacter* negative group.	*Campylobacter* positive group had a significantly higher abundance of *Bacteroides* and *Escherichia* species than those in *Campylobacter* negative group

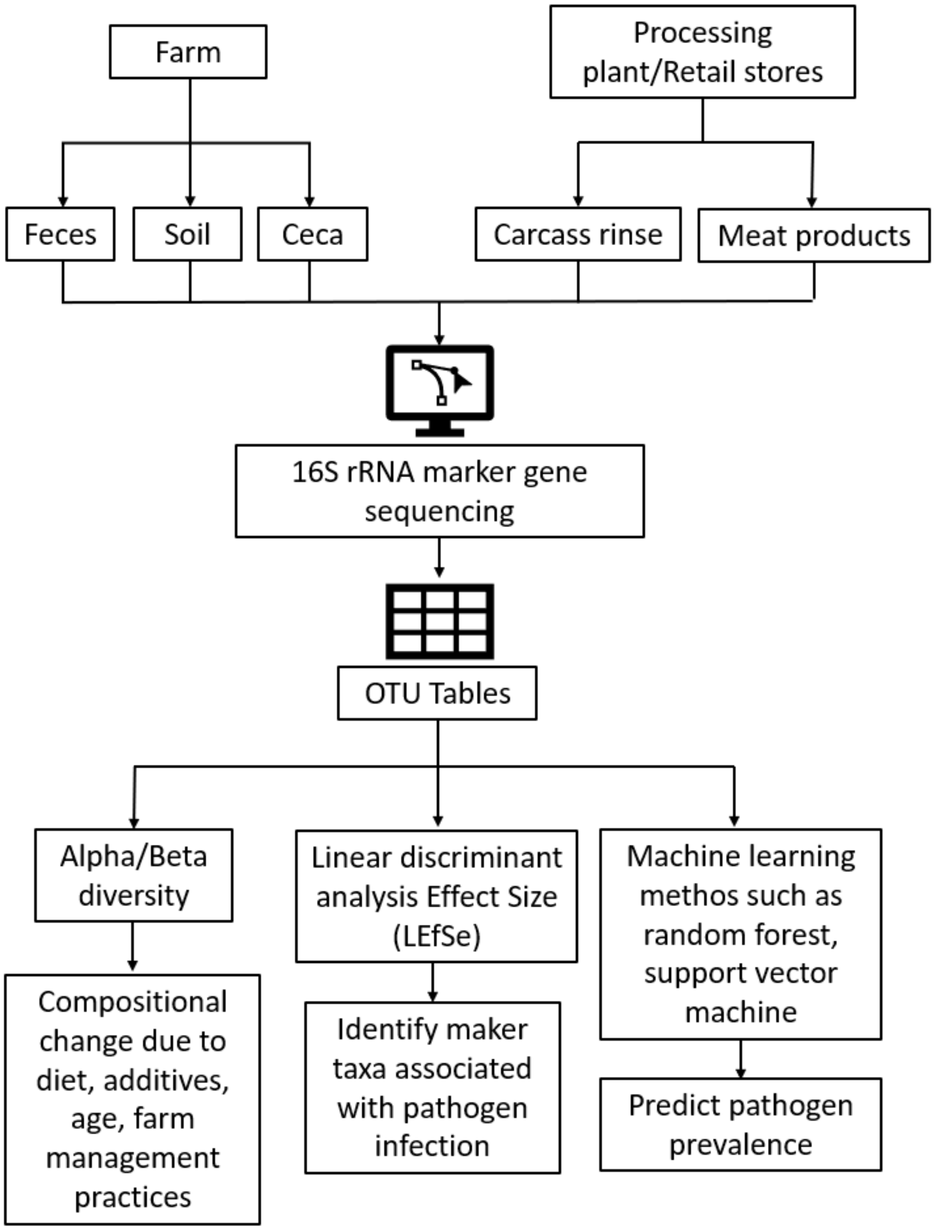

Figure 2.1 Flow diagram of microbiome analysis

CHAPTER 3

COMPARISON BETWEEN LASSO AND RT METHODS FOR PREDICTION OF GENERIC *E. COLI* CONCENTRATION IN PASTURED POULTRY FARMS[1]

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[1] Xu, X., Rothrock Jr, M. J., Reeves, J., Kumar, G. D., & Mishra, A. (2022). *Food Research International*, 161, 111860. Reprinted here with permission of the publisher.

Abstract

Though most strains of *E. coli* are non-pathogenic components of the intestinal microbiome, certain pathogenic *E. coli* strains are the cause of diseases and outbreaks. Poultry is identified as a common reservoir for pathogenic *E. coli*. It is important to identify farm practice factors associated with *E. coli* in the pastured poultry environment. The objective of this study is to develop models that can predict *E. coli* levels and to select farm practice factors contributing to *E. coli* concentration in pastured poultry farms. Five kinds of samples: feces, soil, whole carcass rinse after processing (WCR-P), final product after chilling and storage (WCR-F), and ceca samples were collected for *E. coli* counts from 11 pastured poultry farms in the southeastern US. The regression tree (RT) and least absolute shrinkage and selection operator (LASSO) methods were applied to data from each sample type. The farm management practices and processing factors such as source of eggs, type of feed used, appearance of other animals on farm, chilling method, and storage time and temperature were all considered as possible explanatory factors in the models. Models were developed to predict the levels of *E. coli* and to select the most important factors used in predicting *E. coli* population. Model performances were compared using root mean square error (RMSE). For feces samples, average number of birds and animal source were the two most important variables affecting *E. coli* population by LASSO. The RT selected animal source, brood feed, day of year, flock age in days, and flock size as the most important variables in predicting *E. coli* concentration. The RMSE (in $\log_{10}$ scale) under LASSO was 0.974, while under RT it was 1.032 for feces samples. The predictive models provide practical and effective tools to predict *E. coli* population and to identify farm practice factors that affect *E. coli* levels.

3.1 Introduction

Escherichia coli is a facultative anaerobic, Gram-negative bacterium which is primarily present in the gastrointestinal tract (GIT) of warm-blooded animals and humans (Donnenberg and Whittam, 2001). Generic *E. coli* is found in the feces and GIT of all meat and poultry animals. An ideal indicator should have similar multiplication rate as the pathogens, should present in feces with high numbers, should be non-pathogenic and be quickly, easily, and economically detected (Ghafir et al., 2008a). The widespread presence and high numbers of generic *E. coli* are suitable characteristics for an indicator organism to monitor process control (Berrang et al., 2008; Ghafir et al., 2008b). As a result, the United States Department of Agriculture (USDA) requires meat and poultry processors to do carcass testing for generic *E. coli*. The testing aims to evaluate the hygiene of the plant's slaughter and dressing procedures. It is also a means of verifying process control. The USDA's hazard analysis and critical control point rule specifies two criteria for evaluating process control: processors are to maintain less than 100 CFU of *E. coli* per ml in 80% of poultry carcass rinses and never exceed 1,000 CFU/ml (Altekruse et al., 2009).

Over the last decade, the transmission routes of *E. coli* to humans have been studied. Kim et al. (2020) summarized three main transmission routes: environment, domestic animals, and wild animals. The increased contact between wild animals and humans due to the adaption of urban exploiter animals reinforced the transmission of pathogenic *E. coli* by feces contamination, the environment, or the water chain (Rothenburger et al., 2017). In addition, a systematic review by Lazarus et al. (2015) presented evidence that poultry is a reservoir for human enteropathogenic *E. coli* (EPEC). Schroeder et al. (2004) reported that the majority of *E. coli* resistant strains were isolated from poultry and traditional retail meats. As a reservoir, *E. coli* may contaminate foods in various ways including processing, handling, indirect contamination with tainted water, and packaging of finished product (Persad and LeJeune, 2014). Manges (2016) reported that *E. coli* has been recovered from waterways, sewage, and domestic, and wild animals, soil, and other environmental samples, suggesting multiple possible transmission pathways to human.

Machine learning (ML) algorithms have been used in food safety, food processing, and quality evaluation (Deng et al., 2021; Du and Sun, 2006). Machine learning is a sub-field of Artificial Intelligence (AI) and can be defined as the field of computer science that gives computers the ability to learn without being explicitly programmed (Kotwal et al., 2021). The computers are programed to learn from input data and learning is depended on the algorithms. The learning process will then convert input information and experience into output outcome such as classification and prediction. ML models for classifying and predicting foodborne pathogens have been in several studies (Golden et al., 2019a; Hwang et al., 2020a), suggesting ML models a promising tool for prediction and monitoring in food safety.

Pastured poultry is a sustainable agricultural method which has grown interest in recent decades. The pastured poultry process raises chicken in movable, open-air pens where pens are rotated to fresh pastures daily or every two days (Rothrock et al., 2019). Broiler chickens are often less expensive compared to pastured raised chickens, but U.S. consumers have shown a willingness to pay more for organic or pastured chicken products (O'Bryan et al., 2015). With the increasing demand for pastured chickens, concerns about food safety have become crucial. Some studies have focused on discussing the relationships between poultry and human infection (Lee et al., 2009; Stromberg et al., 2017). Some other studies have investigated the presence of foodborne pathogens in pastured poultry farms (Golden et al., 2019b; Hwang et al., 2020b; Xu et al., 2021; Xu et al., 2022). However, the study of *E. coli* transmission in pastured poultry farms is very limited, which may lead to economic loss to farmers and potential health risks to consumers. The current study evaluates feces and soil samples collected from pastured poultry farms and chicken carcass rinse from processing facilities. These results can help risk assessors estimate *E. coli* concentration at the farm level. Further, these results can be integrated into risk assessment models.

3.2 Materials and Methods

3.2.1 Sample collection

Feces, soil, chicken carcass rinse, and ceca samples were collected from 42 flocks in 11 pastured poultry farms in the southeastern U.S. from March 2014 to November 2017. Samples were not collected in December, January, and February because there was no operation in winter on pastured poultry farms. In this study, all broilers reared in the 11 farms were pastured chickens. Data on 41 major pastured farm practice variables were recorded over a flock's lifecycle and all samples were evaluated for the counts of *E. coli* level in $\log_{10}$ CFU/g. Five types of samples were collected from each flock: (i) feces, (ii) soil, (iii) whole carcass rinse directly after processing (WCR-P), (iv) final product whole carcass rinse after chilling and storage time (WCR-F), and (v) ceca. For multi-use farms that raise pastured layers, swine, or cattle, feces and soil samples were also collected from the area where these animals resided at the time of sampling. Feces and soil samples were taken three times throughout a flock's lifecycle: (i) within a few days of being placed on pasture, (ii) halfway through their time on the pasture, and (iii) on the day the flock was processed. In total, 2,305 samples were collected, and ten samples showed no growth and were excluded in analysis. There were 814 feces samples, 810 soil samples, 235 WCR-P samples, 230 WCR-F samples, and 206 ceca samples. The explanatory variables are listed in Table 3.1. Specifically, feces and soil samples included 32 variables. WCR-P, WCR-F, and ceca samples included 9 variables which are specific for processing samples in addition to the 32 variables. In total, models for WCR-P, WCR-F, and ceca samples included 41 variables.

On each sampling day, samples were taken from the area where chickens resided. The sampling site was divided into five sections, where five subsamples were collected and pooled from each section to create one sample. Feces samples were collected by sampling fresh fecal droppings on the sampling site. Soil samples were collected by scooping topsoil (approximately 0-7 cm from the surface) into sterile bags. Sterile scoops were used for each sample, and scoops and gloves were changed after each sample. All pooled samples were at least 25 g in weight. Samples were immediately transferred on ice to a laboratory for processing.

On the day of on-farm processing, ceca and carcass rinse samples were collected. Cecal sacs from five carcasses were removed and placed into a single sampling bag to create a pooled sample after evisceration. A total of five pooled samples (n = 5) was created. Gloves and scissors were changed between each pooled sample. For the WCR samples, 100 mL of 10 mmol L^{-1} PBS was added to each carcass within the storage bag, and the bags were vigorously shaken for 60 s. Five WCRs were pooled into a single filtered stomaching bag, and this was repeated five times (n = 25 carcass rinses). No further dilution in 10 mmol L^{-1}PBS was required for the WCR samples. All samples were homogenized for 60 s, and these homogenates were used for all downstream cultural isolations.

3.2.2 *E. coli* enrichment and isolation

Recovery of *E. coli* was performed by spreading 1 mL of the homogenates onto Petrifilm *E. coli*/Coliform Count Plates (3M, MN, USA) which was then incubated overnight at 37 °C. Blue colonies with associated gas production indicative of *E. coli* were identified, and up to five colonies per sample were isolated and used for further characterization. The zeroes in our results were caused by the fact that, when using $\log_{10}$ CFU/g as the unit, $\log_{10}$ of one is zero when we obtained one colony on the least diluted plates. The appearance of colony meant the sample was positive. We use one as the value. For the samples that did not have any growth on plates, we assigned NA (not available)

3.2.3 Statistical analysis

All statistical analyses were performed in R (version 3.4.0; R Foundation for Statistical Computing, Vienna, Austria). One-way analysis of variance (ANOVA) was performed to detect the difference between sample types. Results with p value less than 0.05 were considered statistically significant. Two statistical methods, least absolute shrinkage and selection operator (LASSO) and regression trees (RT) were used to perform feature selection or to determine rules that may have important effects on *E. coli* population in the environmental and chicken carcass rinse samples. The *E. coli* concentration ($\log_{10}$ CFU/g) was used as the response variable in statistical analysis. Models of LASSO and RT methods were built separately based on sample types.

LASSO is a regression analysis method that performs both variable selection and regularization to enhance the prediction accuracy and interpretability of the model (Tibshirani, 1996). It is especially useful when a large number of potential explanatory variables are being considered, as is the case here. The goals of LASSO regression are to identify the variables and their corresponding coefficients that minimize the function $\sum_{i=1}^{n}(y_i - \sum_{j=1}^{p} x_{ij}\beta_j)^2 + \lambda \sum_{j=1}^{p} |\beta_j|$. In this function, $\sum_{i=1}^{n}(y_i - \sum_{j=1}^{p} x_{ij}\beta_j)^2$ is the residual sum of squares and $\lambda \sum_{j=1}^{p} |\beta_j|$ is the sum of the absolute value of the magnitude of coefficients, y_i is the observed value, x_{ij} is the explanatory variables, β_j is the coefficients, and λ is the penalty parameter. The minimization of this function is achieved by penalizing the coefficients of candidate predictors, shrinking some of the unimportant coefficients to zero and thus achieving variable selection (Ranstam and Cook, 2018).

The parameter λ controls the strength of penalty. When λ is zero, it implies that all features are considered, and it is equivalent to the linear regression where only the residual sum of squares is considered to build a predictive model. When λ is very large, or infinity, it implies that no feature is considered. As the value of λ increases, more coefficients will be set to zero resulting in fewer variables being selected. The data sets were divided into training and testing sets. The penalty parameter λ was selected with minimum mean square error by ten-fold cross validation using training set. The selected λ was then used to build the model and to obtain the coefficients for selected explanatory variables. The predicted values were obtained from the model and were compared with the observed values in testing set. Root mean square error (RMSE) was calculated to assess model fitting. The R package *glmnet* was used for LASSO model fitting (Friedman et al., 2010).

In addition to LASSO method, the stability selection algorithm was used to identify significant explanatory variables (Meinshausen and Buhlmann, 2010). This is achieved by repeating the LASSO 100 times to a random subset of the data of size n/2. In every iteration, the same λ that is identified in the previous step is used and the identified variables are recorded. At the end of iteration, the selection probability of each variable is calculated by the number of times selected divided by the number of iterations. The variables appear most frequently in the subsamples are selected. Variables with selection probability close to 1 are the most important. The typical suggested cut-off for deciding whether a feature is important or not is 0.6

(Meinshausen and Buhlmann, 2010). The R package *stabs* was used to perform stability selection analysis (Hofner et al., 2015).

The second method used was regression trees (RT) (Breiman, 1996), which is an algorithm based on decision trees. A decision tree is a flowchart structure, where each node denotes a test on an attribute, whereas each branch represents an outcome of the test. Each tree explains variation of a single response variable (categorical or continuous) by one or more explanatory variables. The tree is developed by repeatedly splitting the data using a simple rule based on a single explanatory variable (De'ath and Fabricius, 2000). At each split, the variables that minimize misclassification error or mean square error are selected. Splitting is continued till an overlarge tree is grown, which is then pruned to a desired size using ten-fold cross-validation. Pruning is done by selecting a tree size that minimizes the cross-validation error. Specifically, the complexity parameter (cp) associated with minimum error is used in ‘prune’ function. However, the tree model selected by cp is too complex. To avoid overfitting, a tradeoff is made to make the model simple with a reasonably higher error. In this case, the model is easy to interpret but still will capture the variables that mostly affect the *E. coli* concentration. The R package *rpart* was used to build and prune the tree. Root mean square error (RMSE) was used to compare the LASSO and RT methods for each sample type:

$$RMSE = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(y_i - \hat{y_i})^2}\ , \tag{2}$$

Where, y_i is the observed value, $\hat{y_i}$ is the predicted value, and n is the sample size.

3.3 Results

The mean *E. coli* levels were different between sample types (Table 3.2). The mean *E. coli* concentration between sample types were significantly different from each other ($p < 0.05$). The feces samples showed the highest mean *E. coli* concentration of 6.46 $\log_{10}$ CFU/g while

WCR-F samples demonstrated the lowest mean *E. coli* level of 1.63 $\log_{10}$ CFU/g. Compared to WCR-P (2.12 $\log_{10}$ CFU/g), the mean *E. coli* levels for soil (4.01 $\log_{10}$ CFU/g) and ceca (5.23 $\log_{10}$ CFU/g) samples were much higher, indicating that *E. coli* levels are higher in the environmental samples. Table 3.2 also summarizes the root mean square error (RMSE) obtained from both methods. The lower RMSE indicated better prediction and accuracy performance. The RMSE of LASSO and RT were 0.974 and 1.032 $\log_{10}$ CFU/g for feces samples while that of soil samples were 1.437 and 1.476 $\log_{10}$ CFU/g for LASSO and RT, respectively. For WCR-P, WCR-F and ceca samples, the RMSE of LASSO model were 0.639, 0.706, and 0.518 $\log_{10}$ CFU/g respectively, whereas the RMSE of RT were 0.526, 0.665, and 0.618 $\log_{10}$ CFU/g respectively. The *E. coli* prevalence were 99.88%, 99.38%, 100%, 100%, and 98.01% for feces, soil, WCR-P, WCR-F, and ceca samples, respectively.

3.3.1 Factors selected by LASSO and RT for feces samples

Table 3.3 summarizes the explanatory variables selected by LASSO and their coefficients for feces and soil samples (The complete list is shown in Appendix A). For *E. coli* levels in feces samples, the average number of birds, egg source, brood cleaning frequency, and pasture housing were selected by LASSO. Among these factors, the stability selection algorithm identified average number of birds and animal source, as the factors contributing the most to the model (Figure 3.1A). The coefficient of breed (red ranger) was 0.435, indicating that using breed red ranger increases the level of *E. coli* concentration by 0.435 $\log_{10}$ CFU/g. In addition, the coefficient of brood soy free (Y) was -0.510, suggesting that not including soy in the diet decreases *E. coli* population on average by 0.510 $\log_{10}$ CFU/g. Animal source, brood feed, day of year, flock age in days, and flock size were selected by the RT as the factors that could best explain *E. coli* concentration in feces samples (Figure 3.2A). Animal source was identified to be the primary explanatory variable under the RT. The predicted *E. coli* concentrations were lower for feces of cattle and swine (5.20 $\log_{10}$ CFU/g) and higher for feces of broiler and layer (6.60 $\log_{10}$ CFU/g). Under the branch of broiler and layer, feces *E. coli* levels could be further explained by brood feed. With the removal of corn from the feed BWO (barley, wheat, oat) and W (wheat), the predicted *E. coli* population were 6.00 $\log_{10}$ CFU/g. This number was lower than the *E. coli* levels applying brood feed contained with corn: CSW (corn, soy, wheat), WC (wheat, corn), CSO (corn, soy, oats), PCO (peas, corn, oats) (6.90 $\log_{10}$ CFU/g). Under this branch, samples collected after the 314th day of the year (second week of November) showed lower *E. coli* population (5.50 $\log_{10}$ CFU/g), while samples collect earlier than day 314 suggested that the *E. coli* concentration were 7.10 $\log_{10}$ CFU/g. For feces samples collected before day 314, flock age greater than 56 days showed the highest predicted *E. coli* levels (7.50 $\log_{10}$ CFU/g). On the other hand, for samples collected earlier than day 314, but with flock age less than 56 days, flock size less than 263 showed higher *E. coli* concentration than that of over 263.

3.3.2 Factors selected by LASSO and RT for soil samples

For soil samples, animal source (swine and cattle) was identified as playing an important role in predicting *E. coli* population by LASSO algorithm (Figure 3.1B). Moreover, the coefficient of brood clean frequency (weekly) was -1.160 which significantly reduced *E. coli* population. On the other hand, egg source D increased *E. coli* concentration by 1.198 relative to other egg sources. Meanwhile, the RT identified animal source, brood cleaning frequency, flock age in days, and pasture feed that significantly explaining *E. coli* levels. In Figure 3.2B, the tree branches show that animal source as the primary rule to classify soil samples. The predicted *E. coli* levels were 1.50 $\log_{10}$ CFU/g for cattle and swine but were 4.40 $\log_{10}$ CFU/g for broilers and layers. Under the section of broiler and layer, brood cleaning frequency with weekly and unknown (NA) showed lower *E. coli* population (3.10 $\log_{10}$ CFU/g) compared to *E. coli* population of cleaning frequency of 3 days, AIAO (all in/all out), daily, DLM (deep litter method), and yearly (4.60 $\log_{10}$ CFU/g). Within the branch of weekly and NA, flock age days over 39 suggested that *E. coli* concentration were 3.50 $\log_{10}$ CFU/g while the *E. coli* levels were 1.60 $\log_{10}$ CFU/g for flock age days less than 39. Pasture feed was identified as a rule to classify samples under the branch of brood cleaning frequency (3 days, AIAO, daily, DLM, and yearly). The predicted *E. coli* population were 4.40 $\log_{10}$ CFU/g for pasture feed applied with BWO, CSW, WC, CSO, and PCO and were 5.20 $\log_{10}$ CFU/g for CMW (corn, cotton seed mill, wheat) and W (wheat). After controlling for brood cleaning frequency and pasture feed, animal source again explained the *E. coli* variation indicating that the *E. coli* population for broiler and layer were 5.90 and 3.40 $\log_{10}$ CFU/g, respectively.

3.3.3 Factors selected by LASSO and RT for ceca samples

The coefficients of LASSO for ceca samples are also listed in Table 3.4 (The complete list is shown in Appendix B). The coefficient of water source from well was 0.16, indicating that private water sources positively impacted *E. coli* population in ceca samples compared to public water sources. Soy-free brood feed negatively affected *E. coli* concentrations as the coefficient was -0.646. By the stable algorithm, brood feed was identified as the top variable. The pruned regression tree for ceca samples is shown in Figure 3.2E. Length of feed restriction before processing was the primary rule affecting *E. coli* population. *E. coli* population were predicted to be 4.90 $\log_{10}$ CFU/g when feed restriction hours was less than 17 h. If the feed restriction hours were more than 17 h, the predicted *E. coli* population were 5.70 $\log_{10}$ CFU/g. Under the branch of feed restriction less than 17 h, the predicted *E. coli* concentration were 4.40 $\log_{10}$ CFU/g for samples with years of farming less than 8 years, while *E. coli* population were 5.20 $\log_{10}$ CFU/g for samples with years of farming more than 8 years. On the other side, the predicted *E. coli* levels were 5.60 and 7.00 $\log_{10}$ CFU/g for the application of brood feed with corn as the primary ingredient (CSW and CSO) and the usage of brood feed with a non-corn primary ingredient (PCO), respectively. Under the branch of corn-based brood feed, the *E. coli* population of flocks older than 63 days were 1.00 $\log_{10}$ CFU/g lower than flocks younger than 63 days.

3.3.4 Factors selected by LASSO and RT for WCR-P samples

The factors and their coefficients obtained by LASSO model for WCR-P samples are also shown in Table 3.4 (The complete list is shown in Appendix A). Farm management factors as well as processing factors were used in LASSO model for these samples. For WCR-P samples, the coefficient of scalder temperature at 65 °C was 0.637 indicating this variable positively affects *E. coli* levels by 0.63 $\log_{10}$ CFU/g. Whether rinse water is chlorinated, egg source, and brood feed is soy free contributed the most in predicting *E. coli* concentration in WCR-P samples by LASSO (Figure 3.1C). However, RT selected whether rinse water is chlorinated, whether brood feed is soy free, brood cleaning frequency, day of year, years of farming, and flock size as rules to predict *E. coli* levels in WCR-P samples. Figure 3.2C shows that whether rinse water is chlorinated was the primary rule to classify WCR-P samples. The usage of rinse water supplemented with organic acid showed that the predicted *E. coli* population were 1.20 $\log_{10}$ CFU/g while the application of chlorinated water, water supplemented with vinegar, non-chlorinated water, and unknown (NA) suggested the *E. coli* population to be 3.00 $\log_{10}$ CFU/g. Within the group of organic acid rinse water, the *E. coli* concentration were 0.80 and 1.30 $\log_{10}$ CFU/g for samples without soy and with soy in brood feed, respectively. When the branch of brood feed is soy free, the day of year earlier than 235 showed higher *E. coli* levels (1.10 $\log_{10}$ CFU/g) compared to *E. coli* levels (0.20 $\log_{10}$ CFU/g) for day of year after 235. On the other side, the brood cleaning frequency of daily, weekly, yearly, and unknown (NA) suggested the *E. coli* population to be 2.50 $\log_{10}$ CFU/g and that of 3 days, AIAO, and DLM indicated the *E. coli* population to be 3.20 $\log_{10}$ CFU/g. Year of farming less than 7 years indicated lower *E. coli* population (2.30 $\log_{10}$ CFU/g) compared to greater than 7 years (3.15 $\log_{10}$ CFU/g) under the branch of brood cleaning frequency of daily, weekly, yearly, and unknown (NA).. Furthermore, the combination of flock size less than 63 and day of year earlier than 255 indicated the *E. coli* concentration to be 2.60 $\log_{10}$ CFU/g.

3.3.5 Factors selected by LASSO and RT for WCR-F samples

For WCR-F samples, the stability selection algorithm of LASSO selected years of farming and type of processing plant as the top factors affecting *E. coli* population (Figure 3.1D). Table 3.4 summarizes the coefficients from LASSO model (The complete list is shown in Appendix A). Relative to other forms of pasture housing, chicken tractor free ranger (CTFR) increased *E. coli* levels by 1.05 $\log_{10}$ CFU/g. Compared to soy-based diet, soy free diet reduced *E. coli* concentration by 0.32 $\log_{10}$ CFU/g. The RT identified years of farming, brood feed, flock size, storage time, and day of year as variables that could best classify *E. coli* population (Figure 3.2D). Years of farming was the primary classifier: samples from farms less than 11 years were found to have *E. coli* population of 1.10 $\log_{10}$ CFU/g, while years of farming more than 11 years were 3.10 $\log_{10}$ CFU/g. Under that branch of years of farming less than 11 years, the predicted *E. coli* population were 0.28 $\log_{10}$ CFU/g for brood feed of CSO and PCO and 1.40 $\log_{10}$ CFU/g for brood feed of BWO, CSW, and W. Within this branch, *E. coli* levels were 1.30 $\log_{10}$ CFU/g for storage less than 21 days and 2.70 $\log_{10}$ CFU/g for storage more than 21 days. On the other hand, for samples from years of farming over 11 years, the *E. coli* population of flock size greater than 43 were lower than that of flock size less than 43.

3.4 Discussion

This study investigated the factors that may affect *E. coli* concentration and further provided LASSO and RT models to predict *E. coli* population in pastured poultry farms and processing plant. The predictive ability of the developed statistical models supported the idea that farm management practices and processing factors could be used relatively easily to obtain information on the level of generic *E. coli*. Further, *E. coli* population could be used as an indication of potential foodborne pathogen contamination. Though, the selected farm management practices and processing factors may be subject to the pastured poultry farms used in this study, this study provides general information on the factors that can affect *E. coli* levels.

Many studies have reported *E. coli* prevalence in chickens. Hossain et al. (2008) reported an *E. coli* prevalence of 63.6% in feces samples of broilers. Islam et al. (2014) reported that *E. coli* were positive for 87.5% litter samples on a poultry farm. A high prevalence of *E. coli* in feces samples (81%) were found by Adenipekun et al. (2015). Blaak et al. (2015) found extended spectrum beta-lactamase (ESBL)-producing *E. coli* prevalent in 81% feces in a broiler farm. The same author also found that ESBL-producing *E. coli* positive in 55% soil samples. Blaak et al. (2015) also reported high *E. coli* levels (7 – 11 and 9 $\log_{10}$ CFU/g) for feces and soil samples. Ercumen et al. (2017) identified *E. coli* prevalence to be 95% in soils collected from chicken raising households. For chicken carcasses, a study carried out in Canada isolated *E. coli* from 99.1% of the 1296 broiler carcasses and the mean *E. coli* level was 2.49 $\log_{10}$ CFU/mL for chicken carcasses rinse (Bohaychuk et al., 2009). Lower *E. coli* prevalence (25%) in retail chicken meat was reported (Akbar et al., 2014). Similarly, Zhao et al. (2001) reported that 38.7% of raw chicken meat were positive for *E. coli*. A much higher *E. coli* prevalence (87.5%) were found in retail broiler meat (Eyi and Arslan, 2012). Overall, *E. coli* prevalence and concentration were high for feces and soil samples, which is in line with our results. For WCR samples, we have much higher prevalence but similar *E. coli* concentration with Bohaychuk et al. (2009). The high variation of WCR samples may be due to environment of processing plant, different types of chilling and washing method used, and any cross-contamination during evisceration or other steps.

3.4.1 Factors affecting *E. coli* populations in feces samples

The study identified that animal source was an important factor affecting the *E. coli* concentration in feces samples by both LASSO and RT models. LASSO model indicated that cattle feces had lower *E. coli* populations compared to layer feces. The RT model suggested that the predicted *E. coli* concentration of feces of cattle and swine were 1.40 $\log_{10}$ CFU/g less than the population of broiler and layer. A previous study carried out in Bangladesh reported that the *E. coli* level was 8.50 $\log_{10}$ MPN/g for chicken feces and 6.80 $\log_{10}$ MPN/g for cow feces (Harris et al., 2016). Another study investigated the concentration of pathogenic *E. coli* O157:H7 in dairy herd and presented the range to be 3 to 5 $\log_{10}$ CFU/g (Zhao et al., 1995). The level of *E. coli* varied by animals indicating that the cross-contamination between different animals on the same pasture may happen. This could be important for farms that raise multiple animals, especially if the animals share common pastures. Day of year was identified as a factor influencing predicted *E. coli* population for feces samples. Samples collected after day 314 (early November) had lower *E. coli* concentrations compared to warmer, summer month (June to September). In a study carried out in Morocco, *E. coli* levels were significantly higher on chicken carcasses during spring/summer (April to September) than autumn/winter (November to March) (Cohen et al., 2007). There may be a seasonal trend of *E. coli* concentration due to high temperature and heavy rain which may aid in the growth and transportation of microorganisms

(Lipp et al., 2001; O'Shea and Field, 1992). The model can only be used for areas with similar climatic conditions when utilizing the day of the year. The ambient temperature or other environmental variables are not available in our analysis. Investigating these variables will help future studies and advance our current understanding of the seasonal effects.

3.4.2 Factors affecting *E. coli* populations in soil samples

Animal source was selected by both LASSO and RT model as the most important factor influencing *E. coli* population in soil samples. On pastured poultry farms, chickens are allowed to graze freely, and their feces are deposited directly onto the ground. Consequently, the *E. coli* levels of soil samples were strongly affected by the type of animal feces dropped. Similarly, Avery et al. (2004) reported that *E. coli* populations on the ground were different for farms containing cattle, sheep, and pig, indicating that *E. coli* concentration of soil samples may be affected by different type of animals reside. A study performed a comparison of *E. coli* population between soil samples with or without animal feces (Ercumen et al., 2017). The results showed that *E. coli* population were 0.54 $\log_{10}$ CFU/g higher in soil samples with animal feces compared to samples without animal feces, indicating a contribution of animal feces to *E. coli* population in soil. Litter is a common transmission route of spreading infectious agents on poultry farms. The frequency of cleaning the brood litter was identified as a factor influencing the *E. coli* concentration in soil samples. For pasture farmers, there is no clear guidance of how often they should clean the brood. It usually depends on the size of flock, the size of brood, and experience. Our results suggest that weekly cleaning of brood yields lower *E. coli* populations compared to other types of cleaning frequency. Flock age was also an influencing factor in explaining *E. coli* variation, with older broilers (over 39 days of age) having higher *E. coli* populations compared to younger birds. The result is in agreement with Northcutt et al. (2003)

that the *E. coli* population increased with the age of broiler. The colonization of generic *E. coli* was observed in early ages of chickens at low levels and the level of *E. coli* increased as chickens grew.

3.4.3 Factors affecting *E. coli* populations in ceca samples

Smith et al. (2007) reported 5.90 $\log_{10}$ CFU/g of *E. coli* for ceca samples, which is in close agreement with our observations. A much higher *E. coli* population (7.00 $\log_{10}$ CFU/g) was observed in an on-farm study of commercial broiler chickens, indicating that the variation may be due to different rearing method (traditional or pastured) (Chinivasagam et al., 2016). The length of feed restriction before processing was identified as the primary factor for classifying *E. coli* population in ceca samples. The target of feed restriction before processing is to reduce the risk of feces contamination during processing (Bilgili and Hess, 1997; Wabeck, 1972). The withdrawal of feed could empty the ingesta in the gastrointestinal tract, thus decreasing the probability of carcass contamination. The length of feed restriction varies from 2 to 24 hours and the optimal feed withdrawal time is between 8 and 12 hours (Wabeck, 1972). Our results reported lower *E. coli* concentrations for chickens that received feed restriction less than 17 hours than for chickens that received feed restriction greater than 17 hours. Studies have shown that longer hours (more than 14 hours) of feed restriction could lead to heavy sloughing due to intestinal cell necrosis (Northcutt et al., 1997), loss of protective mucus layer (Thompson and Applegate, 2006), and increased probability of foodborne pathogens attaching to intestinal epithelia (Burkholder et al., 2008). In combination with our results, we suggest the length of feed restriction be limited to 14 hours or less.

3.4.4 Factors affecting *E. coli* populations in WCR-P samples

The average *E. coli* levels for our WCR-P samples were 2.12 $\log_{10}$ CFU/g, while Russell (2003) reported an average *E. coli* population of 2.01 $\log_{10}$ CFU/g in WCR samples and Smith et al. (2007) found the mean *E. coli* population for chicken carcasses before chilling were 3.60 $\log_{10}$ CFU/g. Whether rinse water is chlorinated was identified as an important factor affecting *E. coli* in WCR-P samples by both LASSO and RT models. In the LASSO model, non-chlorinated rinse water yielded higher predicted *E. coli* levels, while rinse water with organic acid added reduced *E. coli* concentrations. The RT model predicted the *E. coli* population of WCR-P samples treated by organic acid water to be 1.20 $\log_{10}$ CFU/g, while the rest of the groups were 3.00 $\log_{10}$ CFU/g (Figure 3.2C). Jimenez et al. (2003) showed that the *E. coli* concentrations were reduced after chilling using 25 ppm chlorinated water in a broiler slaughterhouse, which agrees with our data. Moreover, a study reported 1.40 $\log_{10}$ CFU/g *E. coli* reduction before and after chilling with 20 ppm sodium hypochlorite (Northcutt et al., 2003). The results indicated that the application of chlorinated rinse water or organic acid rinse water in broiler slaughterhouses could effectively control *E. coli* populations.

Broilers fed soy-based diets showed a lower predicted *E. coli* population compared to broilers with soy-free diets. Whereas Xu et al. (2021) reported that soy-based brood feed showed higher *Campylobacter* prevalence in soil samples on pastured poultry farms compared to soy-free brood feed diet, although these soy feed differences were not observed on a bacterial community level (Lourenco et al., 2019). The impact of soy-based diet on the presence of indicator organisms and pathogens in broilers requires more research.

3.4.5 Factors affecting *E. coli* populations in WCR-F samples

The generic *E. coli* population on the final product was low in general. In a study conducted in India, *E. coli* population of broiler chicken meat was 2.38 $\log_{10}$ CFU/g (Kumar et al., 2014), which was higher than what we obtain for WCR-F samples (1.63 $\log_{10}$ CFU/g). Similar results were reported that *E. coli* levels of post-chill conventional chicken rinses were 1.70 $\log_{10}$ CFU/mL (Northcutt et al., 2006). Day of year was a factor influencing *E. coli* population in WCR-F samples. The predicted *E. coli* population were high in early June to middle July, which agrees with a study conducted in Australia that the probability of detecting *E. coli* in a sample was significantly higher in summer than in autumn ($p = 0.036$) (Vangchhia et al., 2018). The trend of detecting higher *E. coli* populations in summer months indicated that temperature may be a key factor in controlling *E. coli* concentration. A proper temperature control of processing plants during summer months may be effective in keeping the *E. coli* populations at low levels.

3.5 Conclusions

In conclusion, our findings highlighted the utility of statistical models for predicting *E. coli* concentration in pastured poultry farms. Animal source was found to be important in predicting *E. coli* populations of feces and soil samples by both LASSO and RT methods. Chlorination status of the processing rinse water and soy content of the brood feed were identified as common predictive factors in estimating *E. coli* concentrations in WCR-P samples by both methods, while years of farming was the common feature selected by both methods for WCR-F samples. The models demonstrated the ability to predict *E. coli* populations based on different sample types in pastured poultry farms. These results can be used in risk assessments, to provide farm practices guidance, and for further studies on the risk associated with the production and consumption of poultry products from pastured poultry farms.

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Table 3.1 Explanatory variables used in LASSO and CART model for feces, soil, processing product whole carcass rinse (WCR), and ceca

Variable	Description	Levels/unit
AvgNumBirds	Average number of birds that the farm handle in 1 year	Numeric
AvgNumFlocks	Average number of flocks that the farm handle in 1 year	Numeric
YearsFarming	Number of years the farm had been operating at the time of sampling	Numeric (years)
EggSource	Source of broiler eggs	6 levels: company A, B, C, D, E, F
BroodBedding	Type of bedding broilers received during brooding	3 levels: pastured based brooder (PB), wood shavings (WS), saw-dust/shredded paper (SDSP)
BroodFeed	Up to top 3 sources of protein in brooding feed	6 levels: barley, wheat, oats (BWO); corn, soy, wheat (CSW); wheat, corn (WC); wheat (W); corn, soy, oats (CSO); peas, corn, oats (PCO)
BrGMOFree	Was the brood feed GMO free?	2 levels: yes (Y), no (N)
BrSoyFree	Was the brood soy free?	2 levels: yes (Y), no (N)
BrMedicated	Was the brood feed medicated?	2 levels: yes (Y), no (N)
BroodCleanFrequency	How often the brooding area was cleaned?	6 levels: 3Days, all in/all out (AIAO), daily, deep litter method (DLM), mobile, weekly, yearly
AveAgeToPasture	Average age broilers were put on pasture	2 levels: 3 weeks, 4 weeks
PastureHousing	Type of pasture housing environment	4 levels: chicken tractor (CT), chicken tractor with fencing (CTF), chicken tractor free ranger (CTFR), chicken tractor with fencing (2 tractors; CTF2)
FreqHousingMove	How often the pasture area was moved?	2 levels: daily, every 2 days
AlwaysNewPasture	Was the pasture always moved to a brand-new pasture area?	2 levels: yes (Y), no (N)
PasturedFeed	Up to top 3 sources of protein in pasture feed	7 levels: barley, wheat, oats (BWO); corn, soy, wheat (CSW); wheat, corn (WC); wheat (W); corn, soy, oats (CSO); corn, cotton seed mill, wheat (CMW); peas, corn, oats (PCO)
PaGMOFree	Was the pasture feed GMO free?	2 levels: yes (Y), no (N)

PaSoyFree	Was the pasture feed soy free?	2 levels: yes (Y), no (N)
PaMedicated	Were broilers medicated while on pasture?	2 levels: yes (Y), no (N)
LayersOnFarm	Were layers present on the farm?	2 levels: yes (Y), no (N)
CattleOnFarm	Were cattle present on the farm?	2 levels: yes (Y), no (N)
SwinOnFarm	Were swine present on the farm?	2 levels: yes (Y), no (N)
GoatsOnFarm	Were goats present on the farm?	2 levels: yes (Y), no (N)
SheepOnFarm	Were sheep present on the farm?	2 levels: yes (Y), no (N)
WaterSource	Water source for broilers during grow-out	3 levels: public, rain, well
FreqBirdHandling	How often chickens were handled on pasture?	2 levels: daily, only if needed (OIN)
AnyABXUse	Were antibiotics ever used on the broilers?	2 levels: yes (Y), no (N)
LengthFeedRestrixProcess	Length of feed restriction before processing	5 levels: 8, 12, 16, 18, 24 (hours)
DayOfYear	Day of the year samples were collected on	Numeric (days)
FlockAgeDays	Age of flock at time of sampling	Numeric (days)
Breed	Breed of broilers used	3 levels: freedom ranger (FR), Cornish cross (CC), red ranger (RR)
Flocksize	Number of birds in the sampled flock	Numeric
AnimalSource	Type of the animals	4 levels: broiler, layer, swine, cattle
ProcessingType[a]	Where the broilers were processed?	2 levels: farm, plant
SkinOnOff[a]	Skin on or off processing facility	2 levels: on, off
ScalderTempC[a]	Temperature of water (°C) used during scalding of birds during processing	7 levels: 55, 60, 63, 65, 71, 82, none
RinseWaterSource[a]	Source of water used for carcass rinsing during process	2 levels: public, well
RinseWaterChlor[a]	Was the rinse water chlorinated?	2 levels: yes (Y), no (N)
ChillingMethod[a]	Type of chilling method used for carcasses after processing	2 levels: water, air

TransportTime[a]	Length of time to transport broilers to processors (if necessary)	4 levels: 0.5, 3, 3.5, 5 (hours)
StorageTempC[a]	Temperature that carcasses were stored before reception by customer	2 levels: -20, 4 (ºC)
StorageTimeD[a]	Amount of time carcasses were stored before reception by customer	Numeric (days)

[a] Variables were used in the WCR-P, WCR-F, and ceca models

Table 3.2 Summary of *E. coli* concentration ($\log_{10}$ CFU/g) and RMSE by sample type

	Feces	Soil	WCR-P	WCR-F	Ceca
Mean	6.46[a]	4.01[b]	2.12[c]	1.63[d]	5.23[e]
Standard deviation	1.21	1.93	1.13	1.36	0.98
Sample size	814	810	235	230	206
RMSE (LASSO)	0.974	1.437	0.639	0.706	0.518
RMSE (CART)	1.032	1.476	0.526	0.665	0.618

[a,b,c,d,e] Different letters means the columns are significantly different from each other.

Table 3.3 Coefficients of LASSO model for feces and soil samples

Feces		Soil	
Selected variables (Level)	Coefficients	Selected variables (Level)	Coefficients
Intercept	6.966	Intercept	5.117
Ave Num Bird	-0.251	Egg Source (A)	0.113
Egg Source (D)	0.120	Egg Source (F)	-0.411
Brood Bedding (WS)	0.100	Egg Source (E)	-0.412
Brood Feed (WC)	-0.184	Egg Source (D)	1.198
Brood Feed (CSO)	0.105	Brood Feed (PCO)	-0.104
Brood Clean Frequency (AIAO)	0.171	Brood Soy Free (Y)	0.771
Brood Clean Frequency (DLM)	0.262	Brood Clean Frequency (AIAO)	0.433
Pasture Housing (CTFR)	0.155	Brood Clean Frequency (Weekly)	-1.160
Breed (RR)	0.435	Ave Age to Pasture (4)	-1.625
AnimalSourceCattle	-1.253	Pasture Feed (W)	0.954
AnimalSourceLayer	0.618	Pasture Soy Free (Y)	-0.453
AnimalSourceSwine	-0.665	Pasture Medicated (N)	-0.259
		Goats on Farm (Y)	0.547
		Frequency Bird Handling (OIN)	-0.804
		Any ABX Use (Y)	-1.380
		Breed (RR)	-1.110
		AnimalSourceCattle	0.221
		AnimalSourceSwine	-0.676

Table 3.4 Coefficients of LASSO model for WCR-P, WCR-F, and ceca samples

WCR-P		WCR-F		Ceca	
Selected variables (Level)	Coefficients	Selected variables (Level)	Coefficients	Selected variables (Level)	Coefficients
Intercept	3.013	Intercept	1.350	Intercept	2.896
Avg Num Birds	-0.144	EggSourceD	-0.143	Avg Num Birds	0.690
Egg Source (B)	0.768	EggSourceB	0.571		
Brood Soy Free (Y)	-0.510	Brood Bedding (WS)	0.300	Egg Source (E)	-0.600
Brood Clean Frequency (AIAO)	0.111	Brood Soy Free (Y)	-0.321	Egg Source (D)	0.832
Brood Clean Frequency (Weekly)	-0.124	Brood Clean Frequency (Weekly)	-0.124	Egg Source (B)	0.266
Pasture Feed (W)	0.445	Pasture Housing (CTFR)	1.055	Brood Feed (CSW)	0.299
LayersOnFarmY	0.124	Source (Rain)	Water - 0.131	Brood Feed (CSO)	-0.245
GoatsOnFarmY	-0.153	Processing Type (Plant)	-0.407	Brood Feed (PCO)	0.419
Any ABX Use (Y)	-0.331	Scalder Temp (65 ℃)	0.334	Brood Soy Free (Y)	-0.646
Scalder Temp (65 ℃)	0.637	Rinse Water Chlor (N)	0.162	Avg Age to Pasture (4)	-0.872
Rinse Water Chlor (NA)	0.901	Transport Time (3)	-0.603	Pasture Feed (W)	0.880
Rinse Water Chlor (OA)	-0.874	Breed (RR)	-0.301	Pasture Feed (PCO)	1.221
				Layer On Farm (Y)	-0.313
				Goat On Farm (Y)	1.286
				Sheep On Farm (Y)	0.551
				Water Source (Well)	0.160
				Rinse Water Chlor (N)	0.746

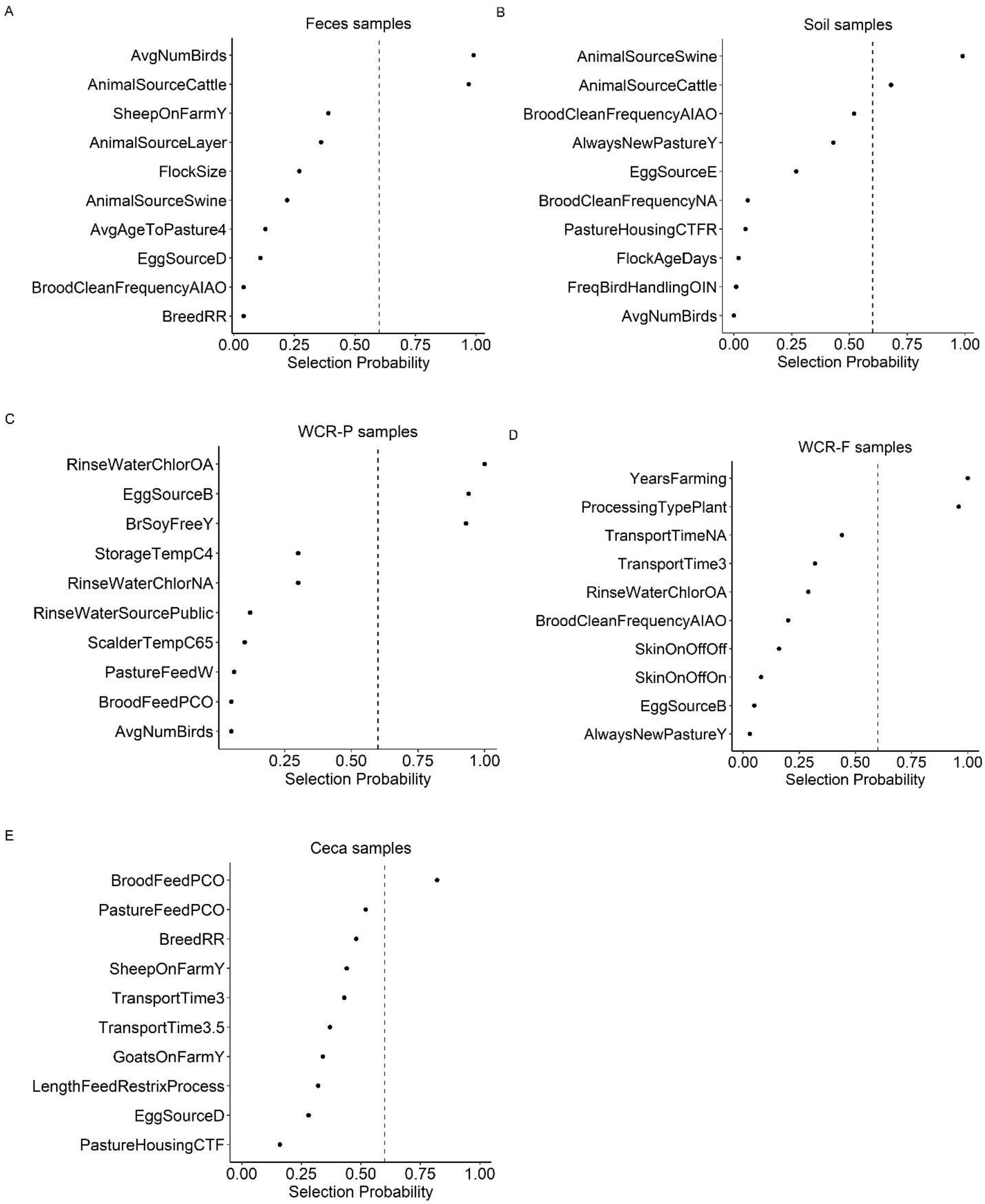

Figure 3.1 LASSO model stability selection of explanatory variables for feces samples (A), soil samples (B), WCR-P samples (C), WCR-F samples (D), ceca samples (E)

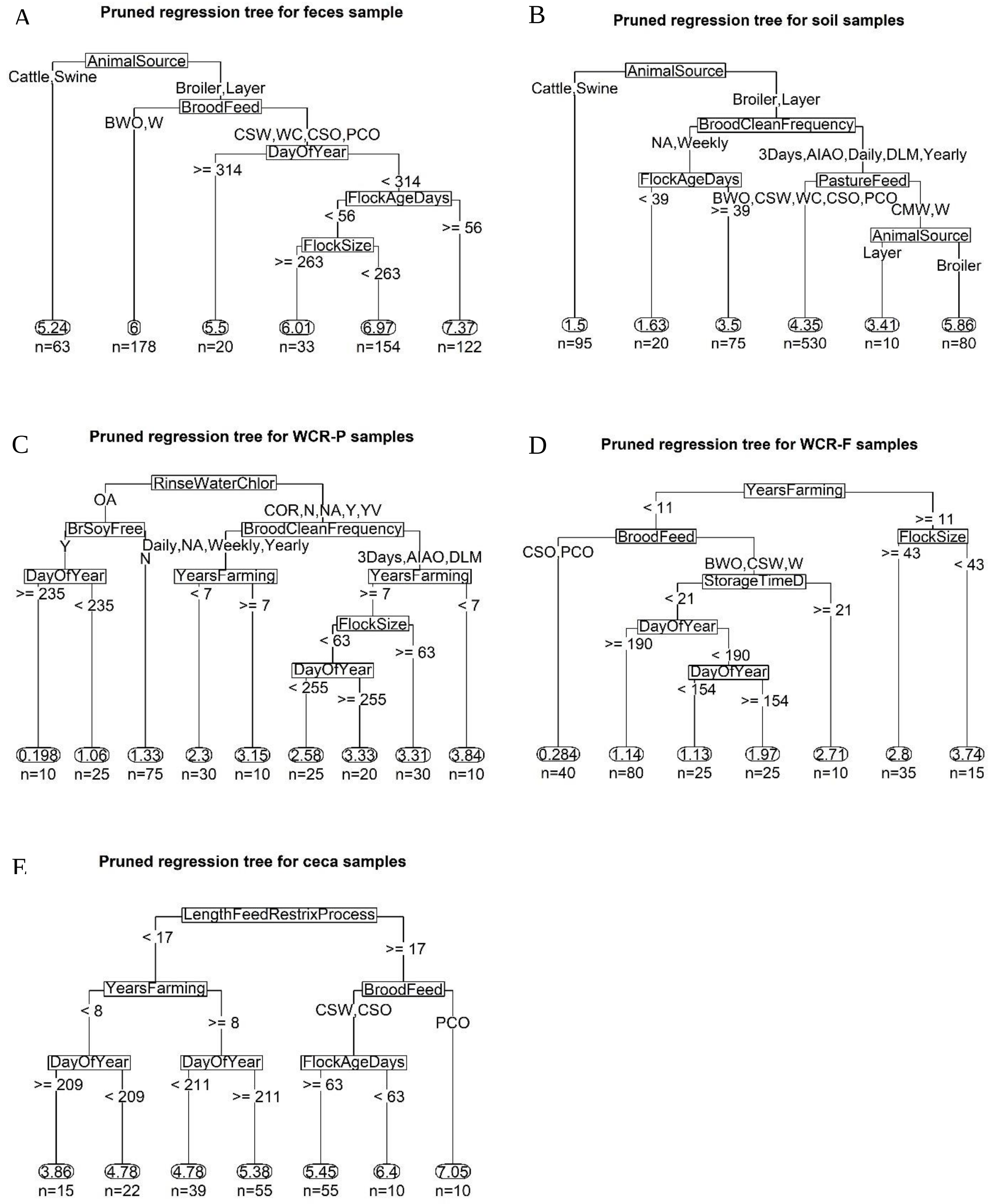

Figure 3.2 Regression tree for feces samples (A), soil samples (B), WCR-P samples (C), WCR-F samples (D), ceca samples (E)

CHAPTER 4

USING FARM MANAGEMENT PRACTICES TO PREDICT *CAMPYLOBACTER* PREVALENCE IN PASTURED POULTRY FARMS[1]

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[1] Xu, X., Rothrock Jr, M. J., Mohan, A., Kumar, G. D., & Mishra, A. (2021). *Poultry Science*, 100(6), 101122. Reprinted here with permission of the publisher.

Abstract

Contamination of poultry products by *Campylobacter* is often associated with farm management practices and processing plant practices. A longitudinal study was conducted on 11 pastured poultry farms in southeastern United States from 2014 to 2017. In this study, farm practices and processing variables were used as predictors for a random forest (RF) model to predict *Campylobacter* prevalence in pastured poultry farms and processing environments. Individual RF models were constructed for fecal, soil and whole carcass rinse after processing (WCR-P) samples. The performance of models was evaluated by the area under curve (AUC) from the receiver operating characteristics curve. The AUC values were 0.902, 0.894 and 0.864 for fecal, soil and WCR-P models, respectively. Relative importance plots were generated to predict the most important variable in each RF model. Animal source of feces was identified as the most important variable in fecal model and the soy content of the brood feed was the most important variable for soil model. For WCR-P model, the average flock age showed the strongest impact on RF model. These RF models can help pastured poultry growers with food safety control strategies to reduce *Campylobacter* prevalence in pastured poultry farms.

4.1 Introduction

The U.S. Centers for Disease Control and Prevention (CDC) estimates that *Campylobacter* causes an estimated 1.5 million illnesses each year in the United States (CDC, 2019). *Campylobacter* spp. are gram-negative spiral, rod-shaped, non-spore forming bacteria with polar flagella (Kaakoush et al., 2015). Major symptoms of *Campylobacter* infection are gastroenteritis, diarrhea, and sequelae such as Guillain-Barre syndrome (Sahin et al., 2003). From 2010 to 2017, among 236 reported foodborne *Campylobacter* outbreaks, 41 were associated with poultry products (CDC, 2019). In addition, the consumption of chicken and eggs linked to *Campylobacter* account for most laboratory-confirmed cases of bacterial gastroenteritis in the United States (Arsi et al., 2019). Poultry is identified as a major reservoir and source of transmission of campylobacteriosis (Kaakoush et al., 2015). After poultry products contaminated with *Campylobacter* are brought to a consumer's kitchen, they can cross contaminate utensils and further infect consumers (Nauta et al., 2009). In general, *Campylobacter* contaminates poultry products before or during processing, surviving through the food supply chain to becoming a potential health risk to humans (Newell and Fearnley, 2003). It is important to identify the factors leading to *Campylobacter* transmission on poultry farms and processing plants. Feed, drinking water, soil, other farm animals, biosecurity threats (wildlife species), insects, farm equipment, employees, visitors, and farm vehicles are possible routes of *Campylobacter* transmission on poultry farms (Ghareeb et al., 2019). Many conventional farm-based studies have explored the sources of flock infection, modes of transmission, and the host and environmental factors affecting the spread of *Campylobacter* (as previously reviewed (Sahin et al., 2002)); however, there is limited information on *Campylobacter* infection in pastured poultry farms. Identification of potential risk factors at the farm level is vital to prevent *Campylobacter* infection in these alternative poultry management systems.

Pastured poultry is a sustainable agriculture technique where chickens have access to fresh pastureland to graze on a daily basis while residing within a moveable pen (Rothrock et al., 2019). Compared with conventional poultry production facilities in which tens of thousands of birds are housed in relatively small areas, pastured flocks have much lower stocking densities (>1.5 ft^2/bird) and have greater access to the farm environment, potentially providing a variety of benefits to the birds, environment, and the consumers. Ponte et al. (2008) reported that the omega-3 fatty acid content in the pastured poultry meat is significantly higher than conventional poultry meat. Karsten et al. (2010) reported an increase of vitamin A, vitamin E and n-3 fatty acid in eggs produced from pastured poultry farms. Health benefits are a key concern for consumers who have shown a rising interest in the pastured poultry products, leading to an increasing demand for pastured poultry products in the U.S. in recent years (Hilimire, 2011). The increased interest in pastured poultry products presents food safety challenges since guidance for pastured poultry growers and scientific research is limited (Elkhoraibi et al., 2017). Karsten et al. (2010) reported that food safety concerns, biosecurity, and a lack of food safety knowledge are the three main challenges faced by pastured poultry growers. Therefore, an increased understanding of food safety issues, and the environmental/management variables that influence food safety issues, is vital to the sustainability of pastured poultry operations.

The environmental factors and farm management practices may have impact on the prevalence of foodborne pathogens in the food products. One method that has been used in the food industry to identify significant environmental or management variables that are correlated to foodborne pathogen prevalence is the use of predictive, machine-learning models. Machine-learning is a set of methods that can automatically detect patterns in data and then use the uncovered patterns to predict outcomes in future data sets (Murphy, 2012). Random forest (RF) model is a commonly used machine-learning method that has been used to predict or track pathogen prevalence in several food safety studies (Golden et al., 2019a; Golden et al., 2019b; Pang et al., 2017; Smith et al., 2010). The RF model is an ensemble of classification and regression trees (B., 2001). After fitting with training data, the prediction is made by averaging the outcome between all the trees. An advantage of this algorithm is its ability to handle complex and high-dimensional data (B., 2001). The goal of this study was to identify farm practices variables associated with *Campylobacter* prevalence in pastured poultry farms using RF machine-learning algorithms.

4.2 Materials and Methods

4.2.1 Sample Collection

A longitudinal study was conducted on 42 flocks of broilers across 11 pastured poultry farms in the southeastern U.S. from March 2014 to November 2017. All 11 farms reared their broiler flocks in movable pens that were moved to fresh pastured daily. The shape, number, and use of temporary fencing around the houses varied among the farms included in the study. A brief description of the size and scale of each farm, as well as other major characterization data, is listed in Table 4.1. Data were collected for 40 major farm practice variables (Table 4.2) over a flock's lifecycle and all samples were evaluated for the presence of *Campylobacter*.

The following samples were collected from each flock to analyze the presence of *Campylobacter*: (i) feces, (ii) pasture soil, (iii) whole carcass rinse directly after processing (WCR-P), (iv) final product whole carcass rinse after chilling and storage time (WCR-F), and (v) ceca samples collected during processing from each farm. If a farm was multi-use and contained pastured layers, swine or cattle (see Table 4.1), fecal and soil samples were collected from the area where these animals resided at the time of sampling. Fecal and soil samples were taken three times throughout a flock's lifecycle: (i) within a few days of being placed on pasture, (ii) halfway through their time on the pasture, and (iii) on the day the flock was processed. In all, 2,305 samples consisted of 815 fecal samples, 815 soil samples, 235 WCR-P samples, 230 WCR-F samples, and 210 ceca samples.

On each sampling day, the moveable pens were moved before sampling, and fecal and soil samples were collected from the areas where the flock was just moved from. The sampling site was divided into five sections, where five subsamples were collected and pooled from each section. On each sampling site, subsamples were pooled for the high variability expected from each subsample and for the possibility that there would be low numbers of *Campylobacter* (Bergholz et al., 2011; Semenov et al., 2008). Fecal samples were collected by sampling fresh fecal droppings on the sampling site. Soil samples were collected by scooping topsoil (approximately 0-7 cm from the surface) into sterile bags. Sterile scoops were used for each sample, and scoops and gloves were changed after each sample. All pooled samples were at least 25 g. Samples were transferred to a laboratory on ice for processing.

4.2.2 Sample Preparation

Upon arrival in the laboratory, samples were prepared as previously described by Rothrock and Locatelli (2019). Briefly, 3 g from each subsample were combined in a filtered stomacher bag (Seward Laboratory Systems, Inc., Davie, FL) and diluted 1:3 with 10 mM phosphate buffered saline (PBS) and homogenized for 1 min. Next, 100 µL of homogenized sample was plated onto Campy-Cefex agar (Neogen, Lansing MI) and incubated at 42 ± 1°C in microaerophilic conditions (85% N_2, 10% CO_2, 5% O_2) for 36-48 h (Stern et al., 1992). Putative *Campylobacter* colonies were enumerated for each plate, and up to 5 suspected colonies were transferred to Brucella agar (Neogen, Lansing MI) supplemented with 10% lysed horse blood (Lampire Biological Laboratories, Pipersville PA) for confirmation and incubated. For model development purposes, samples were classified as positive if countable colonies were found during Campy-Cefex plating.

4.2.3 Model Development

Random forest models were developed for fecal, soil and WCR-P sample data to predict the presence or absence of *Campylobacter* based on the predictors presented in Table 4.2. As the data for WCR-F and ceca samples were extremely imbalanced, random forest models were not developed. For the WCR-P model, predictors specified for processing samples were included where non-processing predictors were used only for fecal and soil models. Before model fitting, each data set was split into training and testing sets. The training and testing sets contained 80% and 20% of the data, respectively. Random forest models were trained with the training set and the test set was served as an independent data set to evaluate the performance of the training model.

4.2.4 Statistical Analysis

All statistical analyses were performed in R (Version 3.4.0; R Foundation for Statistical Computing, Vienna, Austria). The Chi-square test and Fisher’s exact test were used to compare the prevalence of *Campylobacter* across different sample types. Results with *p* value less than 0.05 were considered statistically significant.

The RF model is an ensemble consisting of a collection of classification trees where each tree is independent identically distributed random vectors and these classification trees vote for the most popular class (B., 2001). The classification trees are built based on bootstrap sampling method using a training set which is constructed for drawing observations from the whole data set one at a time and returning them after they have been chosen (Efron, 1979). At each split, the training set is a random subset of all variables, which is a successful approach for assembling unstable leaners (Breiman, 1996; Hastie et al., 2001). With the combination of bagging (bootstrap aggregation) and random variable selection for tree building, each tree in the RF model is unpruned, low-bias tree resulting in low correlation of the individual trees (Diaz-Uriarte and Alvarez de Andres, 2006). However, RF model based on a classification and regression tree (B., 1984) is biased when selecting the important variables, and it favors continuous variables and variables with many categories (Strobl et al., 2007). Strobl et al. (2008) provided ‘cforest’ function to address the issue, which is based on unbiased conditional inference trees by substituting bootstrap sampling with sampling without replacement (Hothorn et al., 2006; Strobl et al., 2007).

The *party* and *caret* package were used for model training and analysis (Hothorn et al., 2010; Kuhn, 2008). All models were built using “cforest” function with “replace = FALSE” and default option “controls = cforest_unbiased()”. To choose the suitable value for m_{try} and n_{tree}, RF models were trained using various m_{try} and n_{tree} values. The values with highest receiver operating characteristic (ROC) statistic were chosen and the chosen values were implemented in the final model. Variable importance was determined using the mean decrease in accuracy. Variables were ranked by relative importance from low to high where the variable with highest value represents the most important variable. Partial dependence plots (PDP) were built for the two most important variables in each model using *pdp* package (Greenwell, 2017).

To address the imbalance of negative and positive observations of soil and WCR-P samples, the synthetic minority over-sampling technique (SMOTE) was used (Chawla et al., 2002). The SMOTE method applies a mix of over-sampling minority class and under-sampling majority class to make a balanced training set. After model construction, test set was used to validate the performance of each model. Models were evaluated using area under the ROC curves (AUC) (Andrew, 1997), sensitivity and specificity.

4.3 Results

Of the 2,305 total samples collected, 910 (39.5%) were *Campylobacter* spp. positive (Table 4.3). For all the samples collected, the five sample types showed significantly different *Campylobacter* prevalence (χ^2 = 728.06; *d.f.* = 4; p < 0.0001). To compare the difference between every two groups, Fisher's exact test was performed. *Campylobacter* prevalence was significantly higher in fecal samples (61.1%), compared to the soil (21.1%, $p < 0.0001$), WCR-P (15.6%, $p < 0.0001$), and WCR-F (2.2%, $p < 0.0001$) samples, but the highest *Campylobacter* prevalence was found in the ceca samples (94.3%, $p < 0.0001$). The only pair-wise comparison without a significant difference in *Campylobacter* prevalence was between soil and WCR-P samples (p =0.0779).

Random forest models were constructed for fecal, soil and WCR-P samples. For the fecal model, the relative importance plot containing the top ten most important predictor variables is illustrated in Fig. 4.1. The model predicted that source of animal feces was the most important variable in predicting *Campylobacter* prevalence in pastured poultry farms. The mean decrease accuracy (MDA) value of animal source was 0.096, compared to 0.025 and 0.019 for average number of flocks and average number of birds a farm handles every year, respectively. No other predictor variables were found to have a MDA value over 0.02. Partial dependency plots (PDPs) were built for animal feces source and average number of flocks (Figure 4.2). As shown in Fig. 4.2A, fecal samples collected from broiler chickens appeared to have the highest probability of *Campylobacter* isolation. Fecal samples collected from cattle and swine had the lowest and the second lowest probability of *Campylobacter* isolation, respectively. Though there was some variance within Fig. 4.2B, the model suggested an increasing trend of isolating *Campylobacter* as the average number of flocks increased.

For the soil RF model, the relative importance plot is shown in Fig. 4.3. The soy content (soy-containing versus soy-free) of the brood feed was ranked as the most important variable to predict *Campylobacter* prevalence of soil samples in pastured poultry farm with a MDA value of 0.093. The second most important indicator was types of pasture housing used when rearing the chickens with a MDA value of 0.058. The PDP plot of brood feed soy content suggested that brood feed without soybeans had a higher probability of isolating *Campylobacter* in soil samples than feed with soybeans (Fig. 4.4A). The model predicted that *Campylobacter* prevalence was higher when a mobile chicken tractor with fencing was used than other types of pasture housing (Fig. 4.4B).

The relative importance plot for WCR-P model showed that flock age (weeks) was the most important variable in predicting *Campylobacter* prevalence in processing carcasses samples. The MDA value of flock age was 0.053 followed by day of the year and average number of flocks as the second and third important predictors with MDA values of 0.047 and 0.045, respectively (Fig. 4.5). Compared to fecal and soil RF models, the MDA values for WCR-P model were lower. As shown in Figure 4.6A, the model predicted *Campylobacter* prevalence was higher when flock age was less than 10 weeks and a drop of *Campylobacter* isolation was observed after 10 weeks. Similarly, the probability of *Campylobacter* isolation was higher during the Spring and Summer months (< 210 days into the calendar year) and a drop was observed around the late Fall/Winter months.

For all three models, the performance was evaluated on a test set separated from the original data set. The test set was not used in training the model but aimed to verify model outcomes. Confusion matrices were generated for the three models and were used to demonstrate the ability of models' prediction and observed results (Table 4.4). Sensitivity is using the correctly predicted positive results divided by the actual positive results. Similarly, specificity represents correctly predicted negatives divided by the true positives. In predicting pathogen prevalence, false negatives cost more than false positives. Thus, sensitivity is of great importance for the models. If the sensitivity of a model is close to one, it means the model has low false negative ratio which indicates good predicting ability of a model. The fecal model showed a sensitivity of 0.8692 and specificity of 0.7705. It suggested that the fecal model correctly predicted 93 positive results out of 107 actual positives whereas 47 negative results were correctly predicted out of 61 true negatives. To further present these results, ROC curves and area under the curve (AUC) were used (Fig. 4.7A, 4.7B and 4.7C). An AUC value close to one indicated a good performance of a model. The soil model had a sensitivity of 0.7407 and specificity of 0.8784. Its AUC value was 0.894 (Table 4.4). For WCR-P model, it obtained a sensitivity of 0.7778 and specificity of 0.8529. The AUC value for WCR-P model is 0.864. Overall, the three models received acceptable sensitivity and specificity values that were above 0.7. In the meantime, the models also achieved AUC values above 0.85.

4.4 Discussion

One of the key aspects in reducing *Campylobacter* infection of broiler chickens is to limit transmission pathways. Flies, water source, feces, wild and domestic animal activities on or near the farm, feed, and employee boots have been identified as possible pathways for *Campylobacter* transmission to broilers in conventional farms in the Netherlands, United Kingdom and United States (Gregory et al., 1997; Hald et al., 2004; Humphrey et al., 1993; Jacobs-Reitsma et al., 1995; Pearson et al., 1993; Shreeve et al., 2000). Moreover, bird-to-bird infection within flocks is another potential path of *Campylobacter* transmission. Shreeve et al. (2000) reported that once *Campylobacter* colonization was first detected in one of the flocks, nearly all flocks tested positive for *Campylobacter* one to eight days later. Similarly, Evans and Sayers (2000) reported that it only took three weeks to spread from 40% of the flocks to over 90%. These findings point out the concerns for alternative systems such as pastured poultry farms that are characterized by the exposure to the natural farm environment. Some studies have analyzed the relationship between environmental samples and carcass rinse samples from broiler chicken flocks on conventional farms (Berghaus et al., 2013; Schroeder et al., 2014; Trimble et al., 2013). More research that relate environmental and carcass rinse samples with pastured poultry farm practices are needed. This study utilized the RF algorithm, a machine learning method used to detect the potential patterns between environmental and carcass rinse samples and pastured poultry farm practices. The results from this study could offer guidelines to pastured poultry growers from both statistical and practical standpoint for their farm operations.

The fecal RF model predicted feces source (broilers, layers, swine, and cattle) as the most important variable in predicting *Campylobacter* prevalence in pastured poultry farms. Broilers have the highest probability for carrying *Campylobacter* whereas swine and cattle samples were least likely to have *Campylobacter* (Fig. 4.2A). Out of the 630 fecal samples taken from broilers, 460 (73%) were *Campylobacter* positive. For 50 swine and 45 cattle fecal samples collected, six and zeros positive samples were isolated, respectively. This was not surprising, since Rothrock Jr et al. (2019) previously showed that *Campylobacter* was part of the core microbiome of the pastured poultry gastrointestinal tract. These results indicate that *Campylobacter* infection of broilers are less likely to be due to other agricultural animals on farm. This is in agreement with another study Jacobs-Reitsma et al. (1995), which reported that *Campylobacter* serotypes from layers, swine, sheep, and cattle were different from the ones isolated from broilers. Similarly, another study that investigated ten broiler farms in United Kingdom showed that fecal samples from dogs, sheep, horses, and mammals were *Campylobacter* negative (Bull et al., 2006), indicating a lack of transmission from these animals to broilers. However, Gregory et al. (1997) reported that broiler houses with cattle nearby all tested positive for *Campylobacter.* This suggests that cattle are the reservoirs that maintain the organism on the farm. Similar results were presented by Zweifel et al. (2008), who reported that identical *Campylobacter* genotypes were isolated from broilers as well as other farm animals (swine, cattle, and layers), indicating their role as pathogen reservoirs. The contradiction of these results can be due to different farm practices implemented at each farm. For example, poor control of personnel movement between infected and non-infected areas could lead to cross-contamination between animals. These findings indicate that on a complicated farm environment, a well-designed biosecurity protocol is necessary. The average number of flocks handled on farm was identified as the second highest important predicting variable for the fecal model. In Fig. 4.2B, average number of flocks ranged from 1 to 16, all showed a high probability of *Campylobacter* isolation. This trend is in agreement with another study (Newell and Fearnley, 2003) which reported that once flocks were infected, *Campylobacter* spread to other flocks rapidly usually within a week.

The soy content of the brood feed was identified as the most important variable in detecting *Campylobacter* for the soil RF model. The soy-free brood feed showed higher *Campylobacter* prevalence compared to brood feed with soybeans (Fig. 4.4A). This suggests that soybean, as one of the main sources of plant protein, can lower *Campylobacter* contamination of pasture soils. The removal of soy from pastured poultry diets has been previously shown to reduce *Campylobacter* abundance in fecal and WCR samples, and it was hypothesized that *Campylobacter* were able to metabolize soy components and hence the removal of this nutrient source in soy-free feed led to a decrease in *Campylobacter* (Lourenco et al., 2019). If soy-free feed decreased *Campylobacter* loads in pastured broiler gastrointestinal tracts, then it is possible that *Campylobacter* is passed through in the feces to the soil, resulting in the higher *Campylobacter* prevalence in the pasture soils of broilers fed a soy-free diet during the brood phase. However, soybean-based diets did not show a significant difference compared to other protein source diets in the occurrence of *Campylobacter* in conventionally-reared broiler chickens (Visscher et al., 2017). Other foodborne pathogen data from this same study has shown brood feed composition as a major management variable. Golden et al. (2019b) studied *Listeria* spp. prevalence on these pastured poultry farms using farm management practices as predicting variables and found brood feed composition to be important. The diet consisted of corn, soy, and wheat showed high probability of isolating *Listeria* spp. Similarly, Hwang et al. (2020) studied *Salmonella* prevalence on these pastured poultry farms, and they also found pasture feed and brood feed important variables in predicting *Salmonella* prevalence. These findings, in conjunction with the findings from this current work, highlight the importance of feed composition during the brood phase in predicting foodborne pathogen prevalence throughout the pre-harvest and post-harvest stages of pastured poultry management. Pasture housing was the second most important predictor in detecting *Campylobacter* in the soil model. Four types of pasture housings, chicken tractor, no ranging (CT), single chicken tractor with fencing (CTF), chicken tractor free range (no fence) (CTFR), multiple chicken tractors within same fencing (CTF2), were used among the 11 farms investigated. CTF and CTFR presented the highest and second highest probability of isolating *Campylobacter*. The different housing systems might have an impact on isolating *Campylobacter*. However, it was difficult to control the factors that might affect *Campylobacter* prevalence given the observational design used in this study. Experiments that control housing systems would need to be performed to better explain and understand the relationships.

Whole carcass rinse samples collected during processing (post-chill, pre-packaging and storage) (WCR-P) were found to have a *Campylobacter* prevalence of 37%, whereas *Campylobacter* prevalence of whole carcass rinse sample final product (WCR-F) was 2.2%.

Effective safety practices can prevent *Campylobacter* from spreading during processing. For WCR-P RF model, flock age (weeks) at sampling and day of the year at sampling were predicted as the top two most important variables in predicting *Campylobacter* prevalence. As shown in Fig. 4.6A and 4.6B, the models indicated that probability of *Campylobacter* isolation was higher in WCR-P samples in broilers processed at an earlier age (8-9 weeks) and then decreased as broiler chickens grow older (10-12 weeks). However, the risk of *Campylobacter* contamination of broiler carcasses increased as the age of broilers increased in a European Union-wide study of 561 slaughterhouses (EFSA, 2010), so these differences may be more related to the breed of chicken (fast-growing Cornish Cross versus slow-growing Freedom Ranger) than the age of the flock.

4.5 Conclusions

In conclusion, three random forest models were generated to predict *Campylobacter* prevalence in fecal, soil, and WCR-P samples collected from 11 southeastern United States pastured poultry farms. Our model identified the type of feces from farm animals as the most important predictors in predicting fecal *Campylobacter* prevalence. Additionally, soy-containing brood feed was associated with higher probability of isolating *Campylobacter* in soil samples. As predicted by the WCR-P model, flock age was the top variable that affected *Campylobacter* prevalence in WCR-P samples. This study showed the use of RF model in predicting bacteria prevalence and the results should assist in identifying factors that are associated with risks of isolating *Campylobacter*. The generated models will help provide pastured poultry growers and processors with recommendations in implementing farm practices when trying to control or reduce *Campylobacter*.

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Table 4.1 Comparison of the 11 all-natural, antibiotic free, pastured broiler farms included in this study

Farm	Breed	No. of flocks	Flock size	Multi-use farm?	Animal type(s)	Processing
A	Freedom Ranger	10	>500	Yes	Layers, swine, cattle, sheep	USDA-inspected plant
B	Freedom Ranger, Cornish Cross	5	<50	Yes	Layers, swine, goats	on farm
C	Freedom Ranger	1	<50	No	NA	on farm
D	Freedom Ranger	1	<50	No	NA	on farm
E	Freedom Ranger, Cornish Cross	5	50-100	Yes	Layers, swine, cattle, sheep	on farm
H	Freedom Ranger	2	>500	Yes	Layers	USDA-inspected plant
I	Freedom/Red Ranger, Cornish Cross,	8	100-500	Yes	Layers, swine, goats	USDA-inspected plant
J	Freedom Ranger, Cornish Cross	2	50	Yes	Layers	USDA-inspected plant
K	Freedom Ranger	4	100-500	Yes	Layers, cattle, goats	on farm & USDA-inspected plant
L	Freedom Ranger	2	>500	Yes	Layers, swine, cattle, sheep	USDA-inspected plant
M	Cornish Cross	2	50-100	Yes	Layers, swine	on farm

Table 4.2 Predictors used in the fecal, soil and processing product whole carcass rinse (WCR) random forest model

Variable	Description	Levels/unit
AvgNumBirds	Average number of birds that the farm handle in 1 year	3 levels: < 1000, 1000 – 10,000, > 10000
AvgNumFlocks	Average number of flocks that the farm handle in 1 year	6 levels: 1, 2, 3, 4, 5, 16
YearsFarming	Number of years the farm had been operating at the time of sampling	2 levels: <10, >=10 (years)
EggSource	Source of broiler eggs	6 levels: company A, B, C, D, E, F
BroodBedding	Type of bedding broilers received during brooding	3 levels: pastured based brooder (PB), wood shavings (WS), saw-dust/shredded paper (SDSP)
BroodFeed	Up to top 3 sources of protein in brooding feed	6 levels: barley, wheat, oats (BWO); corn, soy, wheat (CSW); wheat, corn (WC); wheat (W); corn, soy, oats (CSO); peas, corn, oats (PCO)
BrGMOFree	Was the brood feed GMO free?	2 levels: yes (Y), no (N)
BrSoyFree	Was the brood soy free?	2 levels: yes (Y), no (N)
BrMedicated	Was the brood feed medicated?	2 levels: yes (Y), no (N)
BroodCleanFrequency	How often the brooding area was cleaned?	6 levels: 3Days, all in/all out (AIAO), daily, deep litter method (DLM), mobile, weekly, yearly
AveAgeToPasture	Average age broilers were put on pasture	2 levels: 3 weeks, 4 weeks
PastureHousing	Type of pasture housing environment	4 levels: chicken tractor (CT), chicken tractor with fencing (CTF), chicken tractor free ranger (CTFR), chicken tractor with fencing (2 tractors; CTF2)
FreqHousingMove	How often the pasture area was moved?	2 levels: daily, every 2 days
AlwaysNewPasture	Was the pasture always moved to a brand-new pasture area?	2 levels: yes (Y), no (N)
PasturedFeed	Up to top 3 sources of protein in pasture feed	7 levels: barley, wheat, oats (BWO); corn, soy, wheat (CSW); wheat, corn (WC); wheat (W); corn, soy, oats (CSO); corn, cotton seed mill,

		wheat (CMW); peas, corn, oats (PCO)
PaGMOFree	Was the pasture feed GMO free?	2 levels: yes (Y), no (N)
PaSoyFree	Was the pasture feed soy free?	2 levels: yes (Y), no (N)
PaMedicated	Were broilers medicated while on pasture?	2 levels: yes (Y), no (N)
LayersOnFarm	Were layers present on the farm?	2 levels: yes (Y), no (N)
CattleOnFarm	Were cattle present on the farm?	2 levels: yes (Y), no (N)
SwinOnFarm	Were swine present on the farm?	2 levels: yes (Y), no (N)
GoatsOnFarm	Were goats present on the farm?	2 levels: yes (Y), no (N)
SheepOnFarm	Were sheep present on the farm?	2 levels: yes (Y), no (N)
WaterSource	Water source for broilers during grow-out	3 levels: public, rain, well
FreqBirdHandling	How often chickens were handled on pasture?	2 levels: daily, only if needed (OIN)
AnyABXUse	Were antibiotics ever used on the broilers?	2 levels: yes (Y), no (N)
LengthFeedRestrixProcess	Length of feed restriction before processing	5 levels: 8, 12, 16, 18, 24 (hours)
DayOfYear	Day of the year samples were collected on	Numeric (days)
FlockAgeWeek	Age of flock at time of sampling	Numeric (weeks)
Breed	Breed of broilers used	3 levels: freedom ranger (FR), Cornish cross (CC), red ranger (RR)
Flocksize	Number of birds in the sampled flock	3 levels: 0 – 100, 100 – 500, > 500
AnimalSource	Type of the animals	4 levels: broiler, layer, swine, cattle
ProcessingType[a]	Where the broilers were processed?	2 levels: farm, plant
SkinOnOff[a]	Skin on or off processing facility	2 levels: on, off
ScalderTempC[ab]	Temperature of water (ºC) used during scalding of birds during processing	7 levels: 55, 60, 63, 65, 71, 82, none
RinseWaterSource[ab]	Source of water used for carcass rinsing during process	2 levels: public, well

RinseWaterChlor[ab]	Was the rinse water chlorinated?	2 levels: yes (Y), no (N)
ChillingMethod[a]	Type of chilling method used for carcasses after processing	2 levels: water, air
TransportTime[ab]	Length of time to transport broilers to processors (if necessary)	4 levels: 0.5, 3, 3.5, 5 (hours)
StorageTempC[ab]	Temperature that carcasses were stored before reception by customer	2 levels: -20, 4 (ºC)
StorageTimeD[ab]	Amount of time carcasses were stored before reception by customer	Numeric (days)

[a] Variables were used in the WCR-P model

[b] Variables were used in the WCR-F model

Table 4.3 Effect of sample type on prevalence of *Campylobacter* spp. in pastured poultry samples

Sample type	No. of samples	No. (%) of positive samples[c]
Fecal	815	498 (61.1) a
Soil	815	172 (21.1) b
WCR-P[a]	235	37 (15.7) b
WCR-F[b]	230	5 (2.2) c
Ceca	210	198 (94.3) d
Total	2305	910 (39.5)

[a] Whole carcass rinse after processing (WCR-P)

[b] Final product whole carcass rinse after chilling and storage (WCR-P)

[c] Different letters represent statistically significant different values when comparing sample types ($p < 0.05$ as determined by the Fisher's exact test)

Table 4.4 Predictive performance of random forest models and the confusion matrix of the models

Models	Predictions	Actual		Sensitivity	Specificity	AUC [a]
		Positive	Negative			
Fecal	Positive	93	14	0.8692	0.7705	0.902
	Negative	14	47			
Soil	Positive	20	17	0.7407	0.8784	0.894
	Negative	7	124			
WCR-P	Positive	7	5	0.7778	0.8529	0.864
	Negative	2	29			

[a] AUC is the area under receiver operating characteristic (ROC) curve

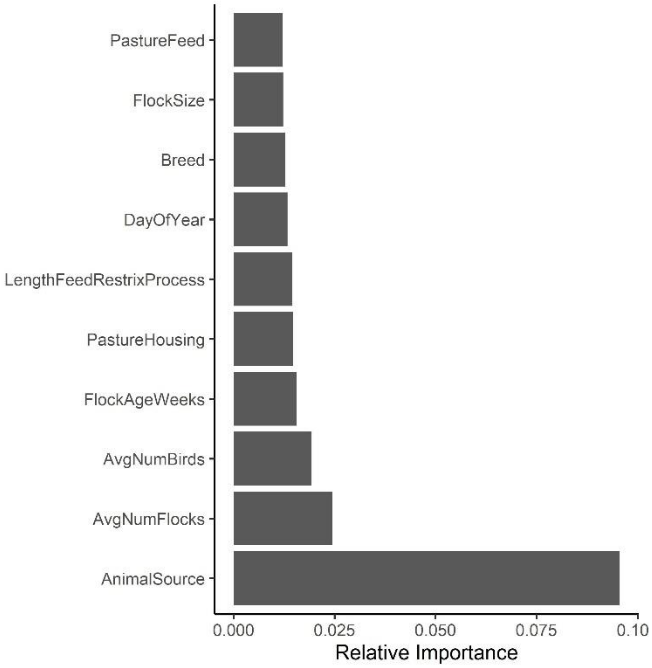


Figure 4.1 Relative importance plot for fecal models

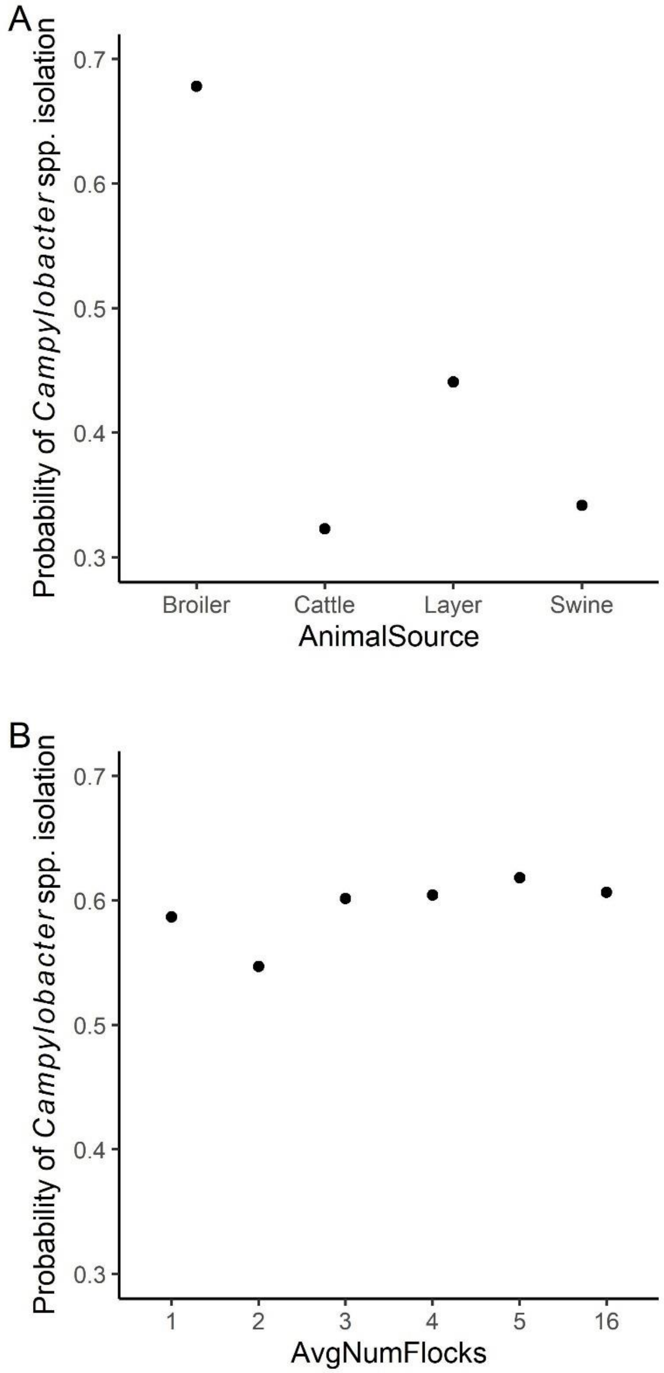


Figure 4.2 Partial dependency plots for the two most important predicting variables in fecal model. Animal source (A) was the most important variable, which represented the types of samples based on animal source, and Average Number of Flocks (B) was the second important variable, which indicated the average number of flocks the farms handle each year

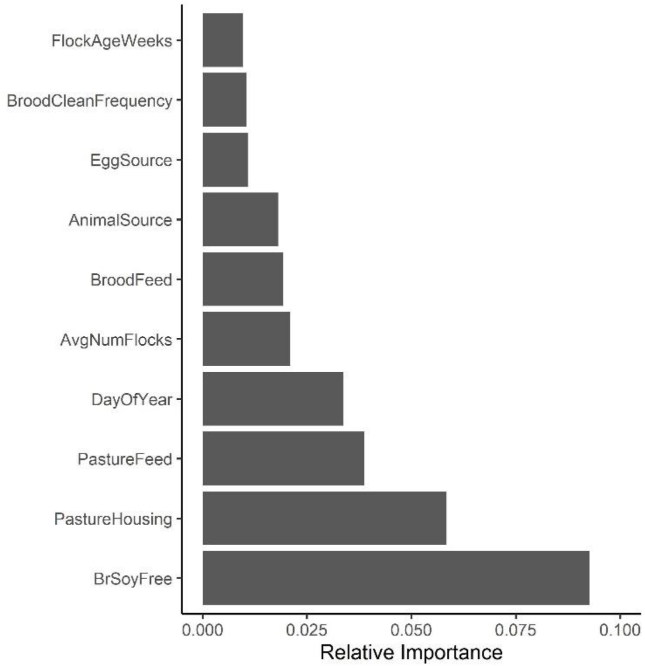


Figure 4.3 Relative importance plot for soil models

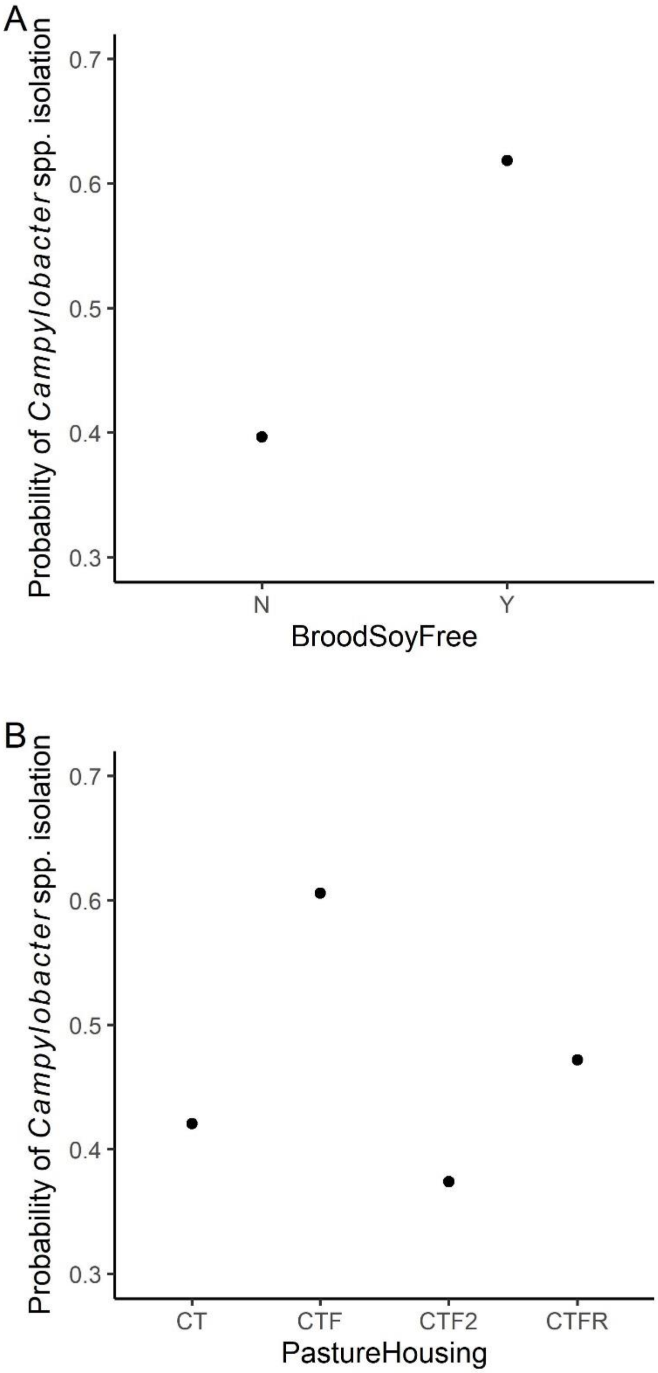


Figure 4.4 Partial dependency plots for the two most important predicting variables in soil model. Brood Soy Free (A) was the most important variable, which represented whether the brood feed is soy free or not, and Pasture Housing (B) was the second important variable, and the types are listed: chicken tractor (CT), chicken tractor with fencing (CTF), chicken tractor free ranger (CTFR), chicken tractor with fencing (2 tractors; CTF2)

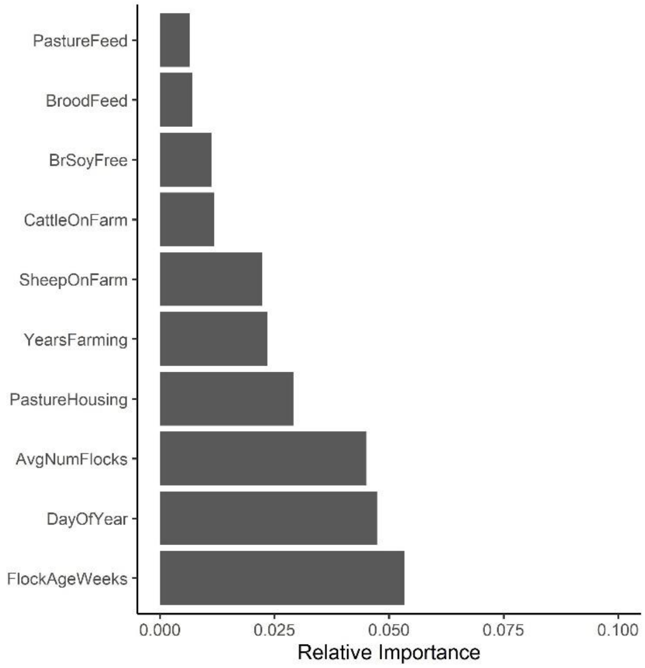


Figure 4.5 Relative importance plot for WCR-P models

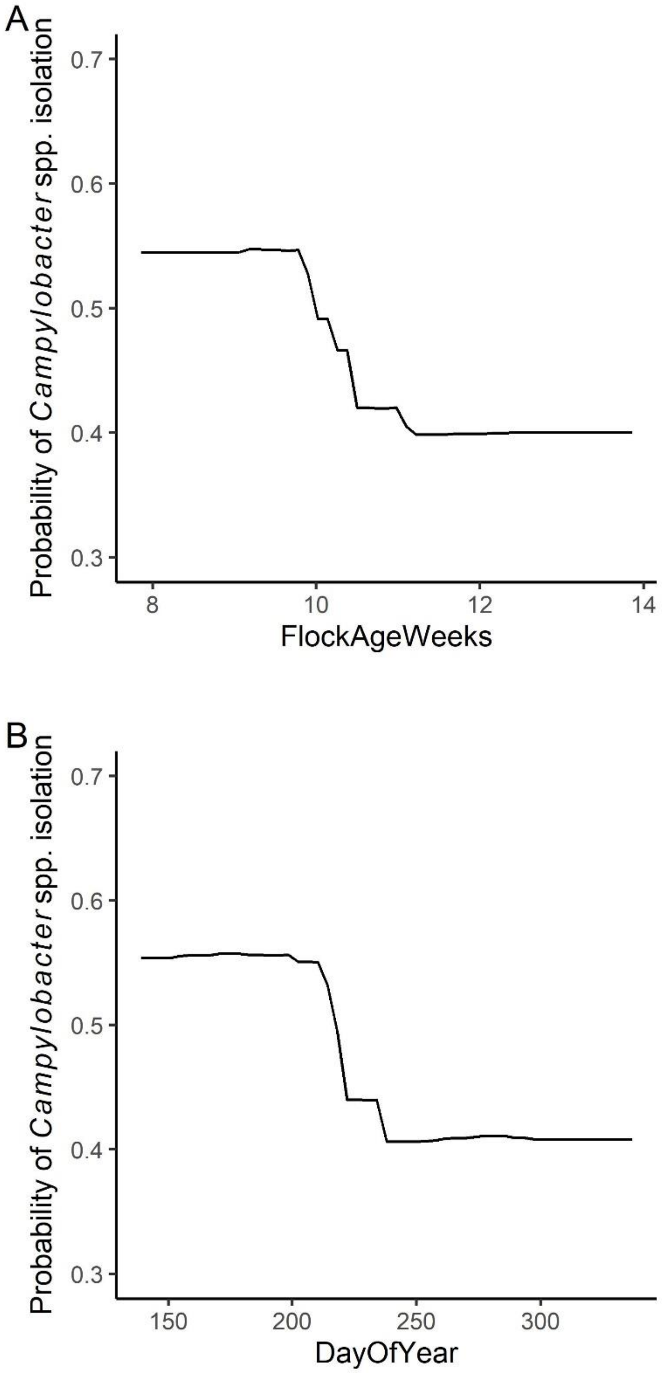
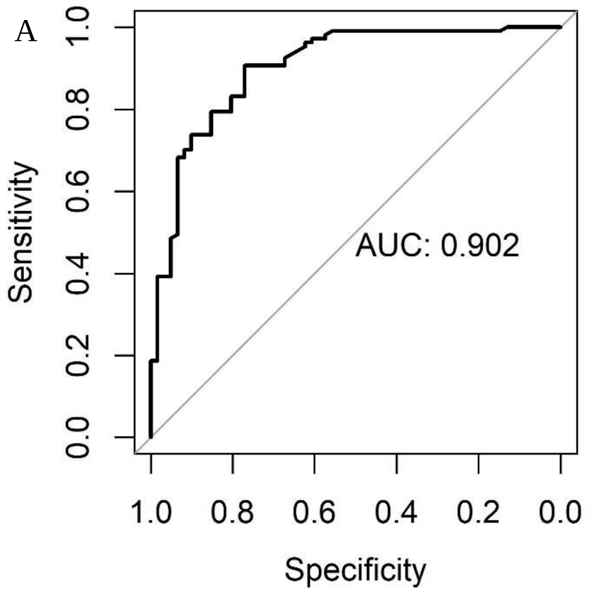
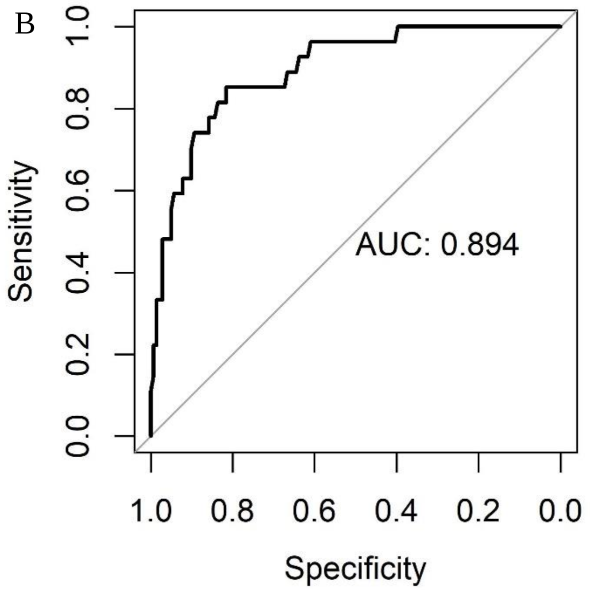


Figure 4.6 Partial dependency plots for the two most important predicting variables in WCR-P model. Flock Age Weeks (A) was the most important variable, which represented the age of a flock at the time of sampling and Day of Year (B) was the second important variable, which means the day of year when samples were collected on


A
Sensitivity
0.0
0.2
0.4
0.6
0.8
1.0
AUC: 0.902
1.0
0.8
0.6
0.4
0.2
0.0
Specificity


B
Sensitivity
0.0
0.2
0.4
0.6
0.8
1.0
AUC: 0.894
1.0
0.8
0.6
0.4
0.2
0.0
Specificity

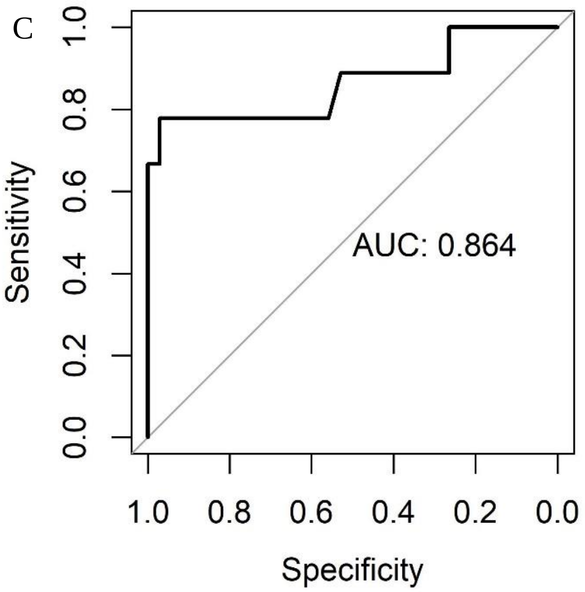


Figure 4.7 Receiver operating characteristic (ROC) curve for A (Fecal model), B (Soil model), C (WCR-P model)

CHAPTER 5

USING *E. COLI* POPULATION TO PREDICT FOODBORNE PATHOGENS IN PASTURED POULTRY FARMS[1]

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[1] Xu, X., Rothrock Jr, M. J., Reeves, J., Kumar, G. D., & Mishra, A. (2022). *Food Microbiology*, 108, 104092. Reprinted here with permission of the publisher.

Abstract

Escherichia coli shows the potential of indicating foodborne pathogens. The purpose of this study is to investigate the association between *E. coli* and foodborne pathogens such as *Campylobacter*, *Salmonella*, and *Listeria* in pastured poultry farms, as well as in related processing facilities. Five different sample types: (i) feces, (ii) soil, (iii) whole carcass rinse during processing (WCR-P), (iv) whole carcass rinse of final product after chilling and storage (WCR-F), and (v) ceca were measured for *E. coli* populations. A logistic regression model for pathogen presence was developed for each sample type. The increase of *E. coli* population significantly increased the predicted probability of *Salmonella* presence for soil and WCR-P samples ($p = 0.0011$ and $p = 0.0157$ respectively). For *Campylobacter*, the initial prevalence in feces and ceca were high and a decreasing trend of detecting *Campylobacter* was observed as *E. coli* concentration increased. In soil and WCR-P models, the probability of the presence of *Campylobacter* significantly increased as *E. coli* population increased. These models provide a practical and effective way of evaluating the relationship between *E. coli* and foodborne pathogens and enable prediction of foodborne pathogen presence based on *E. coli* prevalence within the pastured poultry farm-to-fork continuum.

5.1 Introduction

In early years of food science research, the routine examination of food or environmental samples for the presence of intestinal pathogens was often a tedious, time-consuming, and difficult task. Thus, in response testing for indicator microorganisms for fecal contamination was adopted, based on the fact that certain non-pathogenic bacteria occur in feces of all warm-blooded animals (Gerba, 2009). An indicator organism is a microorganism that is indicative that a food has been exposed to risky conditions; the food may be contaminated with a pathogen, or the food may be held under conditions conducive for pathogen growth (Busta et al., 2003). Indicator microorganisms can be divided into four groups as those that: 1) those that assess the numbers of microorganisms, 2) indicate fecal contamination or possible presence of pathogens, 3) indicate post-heat processing contamination, and 4) indicate metabolic products of pathogens (NRCSMC, 1985). Currently, *Escherichia coli* is the best indicator of fecal contamination among the commonly used fecal indicator organisms (Motlagh and Yang, 2019). Further, the potential of using *E. coli* to indicate presence of foodborne pathogens such as *Salmonella*, *Campylobacter*, and *Listeria* in poultry industry is of interest. These pathogens are commonly associated with outbreaks in chicken and are responsible for causing millions of cases of infection (CDC, 2019). However, one drawback of using indicator microorganisms is that the absence of *E. coli* does not assure the absence of foodborne pathogens.

E. coli are facultative anaerobic Gram-negative bacteria that are found in the intestinal tracts of nearly all warm-blooded animals and humans. Among the six to eight million cases of uncomplicated urinary tract infections (UTI) that occur in the United States every year, over 80% are associated with *E. coli* (not all isolates are pathogenic) (Hooton, 2012). *E. coli* is a major cause of neonatal meningitis (Bonacorsi and Bingen, 2005), gastroenteritis (Scavia et al., 2008), and nosocomial septicemia (Rodriguez-Bano et al., 2010). Though most strains of *E. coli* are normal components of intestinal flora, certain pathogenic *E. coli* strains are cause of disease and outbreaks. There are six distinct groups of pathogenic strains of *E. coli*: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), diffuse-adhering *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), and enterohemorrhagic *E. coli* (EHEC) (Meng et al., 2012).

The potential of using *E. coli* as an indicator microorganism has been discussed in several studies (Belluco et al., 2016; Pacholewicz et al., 2015; Roccato et al., 2018). Indicator microorganisms are markers whose prevalence points to failure to comply with applying Good Manufacturing Practices. These markers can be used to: assess the adequacy of bacterial decontamination treatments to indicate failure or success of the process; assess the hygienic status of the production and processing environment; assess the risk of post-processing contamination; and assess the overall quality of foods (NRCSMC, 1985). Truchado et al. (2018) studied the correlation between *E. coli* and foodborne pathogens and concluded that *E. coli* levels above 2.35 log CFU/ml can be used to predict Shiga toxigenic *E. coli* and *Salmonella* spp. in surface irrigation water. Another study reported that *E. coli* could be used as an indicator microorganism for predicting the level of *Campylobacter* contamination in post-chill broiler carcasses (Habib et al., 2012).

Pastured poultry, as a sustainable agriculture technique, has become more common in recent decades. Pastured poultry is a method to raise chicken in movable, open-air pens where pens are rotated to fresh pastures daily or every two days (Rothrock et al., 2019). Though broiler chickens are often less expensive compared to pastured raised chickens, U.S. consumers have shown a willingness to pay more for organic or pastured chicken product (O'Bryan et al., 2015). Recent studies have focused on revealing the linkage between the presence of foodborne pathogens and farm management as well meteorological factors in pastured poultry farms (Golden et al., 2019a, 2019b; Hwang et al., 2020a; Hwang et al., 2020b; Xu et al., 2021). The correlation between *E. coli* and foodborne pathogens has been studied under several conditions such as pig slaughterhouse, conventional broiler carcasses, and home kitchen (Borrusso and Quinlan, 2017; Nauta et al., 2013; Roccato et al., 2018); however, the study of *E. coli* association with foodborne pathogens in pastured poultry farms is very limited (Thanissery et al., 2012; Trimble et al., 2013). Understanding the presence of *E. coli* and its associated pathogens in pastured poultry farms is important to pastured poultry farmers, as well as consumers. This current study evaluates environmental samples collected from pastured poultry farms and their processing environments. The aim of this study is to measure the association between *E. coli* concentration and three foodborne pathogens (*Salmonella*, *Campylobacter,* and *Listeria*) in these samples.

5.2 Materials and methods

5.2.1 Sample collection and preparation

Eleven pastured poultry farms in the Southeastern U.S. were investigated by collecting samples from 42 flocks of chickens from March 2014 to November 2017, where all chickens reared in the 11 farms were pastured chickens. In total, 1,690 samples were collected, consisting of 629 feces samples, 625 soil samples, 235 WCR-P samples, 230 WCR-F samples, and 206 ceca samples. Five types of samples were collected from each flock for enumeration of *E. coli*: (i) feces, (ii) pasture soil, (iii) whole carcass rinse directly after processing (WCR-P), (iv) final product whole carcass rinse after chilling and storage time (WCR-F), and (v) ceca samples collected during processing from each farm. Feces and soil samples were taken three times throughout a flock’s lifecycle: (i) within a few days of being placed on pasture (16-47 day of age), (ii) halfway through their time on the pasture (36-69 day of age), and (iii) on the day the flock was processed (55-97 day of age). If a farm was multi-use (i.e., contained swine, cattle, or pastured layers, in addition to broilers), feces and soil samples were collected from the area where these animals resided at the time of sampling.

On each sampling day, samples were taken from the area where chickens resided. Each sampling site was divided into five sections, with a subsample collected from each section and the subsamples later pooled. Feces samples were collected by sampling fresh fecal droppings on the sampling site. Soil samples were collected by scooping topsoil (approximately 0-7 cm from the surface) into sterile bags. Sterile scoops were used for each sample, and scoops and gloves were changed after each sample. All pooled samples weighed at least 25 g. Samples were placed on ice and immediately transferred to a laboratory for processing.

During the on-farm processing day, ceca and carcass rinse samples were collected. Upon evisceration, cecal sacs from five carcasses were removed and placed into a single sampling bag to create a pooled sample. A total of five pooled samples (n = 5) was created. Gloves and scissors were changed between each pooled sample. For WCR-P and WCR-F samples, 25 carcasses were sampled after processing, packaging, and cold storage of the carcasses according to the practices followed by each farm. Each carcass was placed in an individual sterile sample bag. Carcasses were rinsed with 100mL of 10mM phosphate-buffered saline (PBS) and the bags were vigorously shaken. Whole carcass rinses from 5 carcasses were combined into 1 pooled sample, creating 5 pooled samples (n = 25) in total. Carcasses were then returned to the processor to be packed, stored, and distributed in the appropriate fashion for that farm.

5.2.2 Cultural enumeration and isolation

5.2.2.1 *Salmonella*

The cultural isolation methods were adapted from Rothrock et al. (2016). Briefly, for pre-enrichment, the stomached homogenates remained in the filtered stomacher bags and were incubated overnight at 35 °C. Two different enrichment broths were used to isolate *Salmonella* from these environmental samples: tetrathionate (TT; Becton-Dickinson, Sparks, MD) broth and

Rappaport-Vassiliadis (RV; Becton Dickinson) media. The 0.5 mL pre-enrichment was transferred into 9.5 mL TT and 0.1 mL pre-enrichment was transferred into 9.9 mL RV. After overnight incubation at 42 °C in both enrichment broths, one loopful (10 μl) from each enrichment broth was streaked on two different differential media: brilliant green sulfa with novobiocin (BGS; Becton Dickinson) agar and xylose lysine tergitol-4 (XLT-4; Becton Dickinson) agar. These plates were incubated overnight at 35 °C, and from each plate, three *Salmonella*-like colonies per subsample were selected and confirmed using triple sugar iron agar (TSI; Becton-Dickinson) and lysine iron agar fermentation (LIA; Becton-Dickinson) using an incubation period of 18-24 h at 35 °C. Final confirmation of suspect TSI/LIA isolates was performed using *Salmonella* polyvalent O antiserum agglutination (Becton-Dickinson), using manufacturer's specifications.

5.2.2.2 *Campylobacter*

Samples were prepared as previously described by Rothrock and Locatelli (2019). Briefly, 3 g from each subsample were combined in a filtered stomacher bag (Seward Laboratory Systems, Inc., Davie, FL) and diluted 1:3 with 10 mM phosphate buffered saline (PBS) and homogenized for 1 min. Next, 100 μL of homogenized sample was plated onto Campy-Cefex agar and incubated at 42 ± 1 °C in microaerophilic conditions (85% N_2, 10% CO_2, 5% O_2) for 36-48 h (Stern et al., 1992). Putative *Campylobacter* colonies were enumerated for each plate, and up to 5 suspected colonies were transferred to Brucella agar supplemented with 10% lysed horse blood for confirmation and incubated. For model development purposes, samples were classified as positive if countable colonies were found during Campy-Cefex plating.

5.2.2.3 *Listeria*

Three g of samples were added to 9 mL of buffered peptone water (BPW; Acumedia, Lansing, MI) in a filtered stomacher bag and shaken vigorously for 30 s. These bags were incubated overnight at 35 °C to serve as a pre-enrichment. Following pre-enrichment, a primary enrichment was made in University of Vermont Modified *Listeria* Enrichment Broth (UVM; Remel, Lenexa, KS) and a secondary enrichment was made in Fraser Broth (FB) (Oxoid CM0895, Basingstoke, UK). Both the primary and secondary enrichments required overnight incubation at 30 °C. One loopful (~10 μL) of the FB enrichment was streaked onto *Listeria* selective agar (LSA; Oxoid CM0856, Basingstoke, UK) for the isolation of *Listeria* spp. colonies. These plates were incubated overnight at 30 °C.

5.2.2.4 *E. coli*

Recovery of *E. coli* was performed by spreading 1 mL of the homogenates onto Petrifilm *E. coli*/Coliform Count Plates (3M) and incubated overnight at 37 °C. Blue colonies with associated gas production indicative of *E. coli* were identified, and up to five colonies per sample were isolated and used for further characterization.

5.2.3 Statistical analysis

All statistical analyses were performed in R (Version 3.4.0; R Foundation for Statistical Computing, Vienna, Austria). The Fisher's exact test were used to test the independence between the presence of *Salmonella* and *Campylobacter*, *Salmonella* and *Listeria*, and *Listeria* and *Campylobacter*. The null hypothesis in each case is that the two pathogens appear independently of one another, while the alternative is that the presence of one affects the presence of the other. Results with *p*-value less than 0.05 were considered statistically significant. In this study, *E. coli* population in $\log_{10}$ CFU/g were used as a predictor variable. The presence and absence (+/-) of

Salmonella, *Campylobacter*, and *Listeria* were response variables. *E. coli* counts data were fitted to logistic regression model using *Salmonella*, *Campylobacter*, and *Listeria* prevalence, as the response by different sample types. The logistic regression model was shown as below:

$$log(\frac{p}{1-p}) = \beta_0 + \beta_1 * E.coli\ population \qquad (1)$$

where p is the predicted probability of identifying *Salmonella*, *Campylobacter*, and *Listeria* respectively; β_0 is the intercept coefficient, β_1 is the slope per unit increase in *E. coli* population.

5.3 Results

Table 5.1 summarizes the sample size, type, pathogen positive rate, and *E. coli* population. The positive rate of *Campylobacter* ranged from 2.17% to 94.14% across different sample types while the positive rate of *Salmonella* varied between 11.48% and 28.51%. For Soil samples, the *E. coli* population ranged from 0 to 8.42 $\log_{10}$ CFU/g with a median of 4.30 $\log_{10}$ CFU/g. The 25% quantile, median, and 75% quantile *E. coli* population were 1.15, 2.21, and 2.99 $\log_{10}$ CFU/g for WCR-P samples. It should be noted that Salmonella and Listeria were isolated via enrichment, so they will have a higher limit of detection than *Campylobacter*, which it was measured via direct plating.

5.3.1 Independence tests between *Campylobacter*, *Salmonella*, and *Listeria*

The *p*-values of these three pairs of tests of independence for all five sample types are shown in Table 5.2. For the pair *Campylobacter* and *Salmonella*, *p*-values of Fisher's exact tests for feces and soil samples are less than 0.05, which indicates that the presence of *Campylobacter* and *Salmonella* are associated. The *p*-values of the pair *Salmonella* and *Listeria* are greater than 0.05 for all sample types expect WCR-F samples, from which it is reasonable to conclude that the correlation between *Salmonella* and *Listeria* are weak in most of sample types. For the pair *Campylobacter* and *Listeria*, *p*-values are less than 0.05 for soil, WCR-P, and ceca samples suggesting that the presence of *Campylobacter* and *Listeria* are dependent on each other in these types of samples.

5.3.2 Logistic regression models for pathogen presence by *E. coli* population for each sample type

Logistic regression was used to predict the probability of presence of *Campylobacter*, *Salmonella*, and *Listeria* based on *E. coli* population. The coefficients of intercept and slope of each sample type are summarized in Tables 5.3, 5.4, and 5.5 for *Campylobacter*, *Salmonella*, and *Listeria*, respectively. The *p*-values of slope of WCR-F and ceca samples were greater than 0.05 suggesting that the increase of *E. coli* concentration did not affect the presence of *Campylobacter* significantly in those two sample types (Table 5.3). In predicting the probability of *Salmonella* presence, *E. coli* population significantly impacted the predicted probability for soil and WCR-P samples (Table 5.4; $p = 0.0011$ and $p = 0.0157$ respectively). For *Listeria*, *p*-values of slope of soil, WCR-P, and WCR-F are less than 0.05, which indicates a significant influence of *E. coli* concentration on the probability of *Listeria* presence (Table 5.5). Moreover, *p*-values of Hosmer and Lemeshow goodness of fit (GOF) test are presented in Tables 5.3, 5.4, and 5.5. Overall, the logistic regression model was judged adequate, with lack-of-fit *p*-values greater than 0.05. The GOF test of *Salmonella* model for soil samples was less than 0.05, indicating that the model was not a good fit. All other models demonstrated a good fit.

5.3.3 Predicted probability of *Campylobacter*, *Salmonella*, and *Listeria* presence based on *E. coli* population

The predicted probability of pathogens over the range of data was plotted against *E. coli* population in Figures 5.1, 5.2, and 5.3 for different sample types. For *Campylobacter* (Fig. 5.1), the chance of identifying this pathogen in feces and ceca samples were 88% and 98%, respectively, at the smallest *E. coli* concentration. The predicted probability of *Campylobacter* presence in feces and ceca samples decreased as *E. coli* concentration increased. Conversely, the predicted probability of *Campylobacter* presence in soil and WCR-P samples increased as *E. coli* population increased. The chance of identifying *Campylobacter* presence in soil samples increased from 10% to 44% while that in WCR-P samples increase from 0% to 53%. The predicted probability of *Campylobacter* presence in WCR-F samples remained low (0.01 to 0.04) and did not vary as *E. coli* concentration increased. In predicting the presence of *Salmonella* (Fig. 5.2), *E. coli* population showed a negative effect on the probability for feces and WCR-P samples. The predicted probability of *Salmonella* presence in WCR-P samples decreased from 0.43 to 0.17 as *E. coli* population increase. Overall, the chance of identifying *Salmonella* was low (ranged from 0.04 to 0.43) and did not vary much as *E. coli* concentration change. For *Listeria* (Fig. 5.3), a sharp increase of chance of finding *Listeria* was observed for WCR-P samples from 1% to 29%. Similarly, the predicted probability of identifying *Listeria* increased from 0.14 to 0.37 as *E. coli* population increase in WCR-F samples. In general, the probability of finding *Listeria* was low (0.01 to 0.37).

5.4 Discussion

5.4.1 Correlation between *Campylobacter, Salmonella,* and *Listeria*

The correlation between *Campylobacter*, *Salmonella*, and *Listeria* was different based on sample types. For feces and soil samples, the presence of *Campylobacter* was significantly associated with the presence of *Salmonella* indicating that *Campylobacter* may be used as an indicator microorganism in such samples. For WCR-F samples, the presence of *Campylobacter* was statistically independent of the presence of *Salmonella*. Similar results have been reported showing no significant correlation between these two pathogens (Hue et al., 2011; Rasschaert et al., 2007).

5.4.2 *E. coli* as an indicator organism for prediction of *Campylobacter* presence

In a study carried out in Italian slaughterhouses, *Campylobacter* populations were found to increase on average by 0.23 and 0.65 $\log_{10}$ CFU/g or every additional log count increase of *E. coli* at post-evisceration and post-chilling for broiler samples, respectively (Roccato et al., 2018). A similar trend was observed in our data that showed a drastic increase of predicted probability of *Campylobacter* presence in WCR-P samples as *E. coli* concentration increased. Conversely, the predicted probability of finding *Campylobacter* in WCR-F samples was low and was not significantly affected by *E. coli* population, while a sharp increase of *Campylobacter* population was observed at the final step of processing by Roccato et al. (2018). Pacholewicz et al. (2015) discussed that *Campylobacter* concentration decreased after scalding and remained the same after defeathering, while the population increased after evisceration and decreased after chilling; however, the patterns of the fluctuation of *Campylobacter* concentration during poultry processing were dependent on slaughterhouses. Similarly, Belluco et al. (2016) and Guerin et al. (2010) reported that the variation of *E. coli* population and presence of *Campylobacter* along the processing lines may be specific to each slaughterhouse. Duffy et al. (2014) presented a strong correlation between *Campylobacter* concentration and *E. coli* concentration along the processing line. On the contrary, Williams and Ebel (2014) reported a weak but significant correlation between *Campylobacter* and *E. coli* at re-hang step. This variation can be partially explained by variations of flocks and by different hygiene and management practices. In a study in Belgium, the median *E. coli* population with and without *Campylobacter* were significantly different in broiler fillet, prepared meat, and layer carcasses but not significant in broiler carcasses (Ghafir et al., 2008). Our data showed that the *E. coli* concentration did not have a significant influence in predicting *Campylobacter* for WCR-F samples, which suggests that further processing (slicing and grinding) of broiler carcasses can potentially increase the chance of introducing *Campylobacter*.

5.4.3 *E. coli* as an indicator organism for prediction of *Salmonella* presence

Chang (2000) tested 27 raw broiler carcasses bought from supermarkets in Korea and found that 7 of 29 (25.9%) tested positive for *Salmonella* presence. For our data, the predicted probability of finding *Salmonella* for WCR-F samples was not significantly different over the range of *E. coli* populations observed, increasing only from 16.8% to 19.9% over the entire range. In comparison, Hue et al. (2011) reported that the *Salmonella* prevalence was 7.5% for broiler carcasses after chilling in a French slaughterhouse, which indicated that the detection of *Salmonella* in final broiler products was relatively low. Jimenez et al. (2002) tested broiler carcasses after evisceration for *E. coli* concentration and *Salmonella*. The mean *E. coli* concentration from *Salmonella*-negative samples and *Salmonella*-positive samples were not significantly different, suggesting *E. coli* may not be a good indicator of *Salmonella*. Conversely, *E. coli* population showed a significant impact on presence of *Salmonella*, with the predicted probability of *Salmonella* presence decreasing as *E. coli* population increased for WCR-P samples. Similarly, Williams and Ebel (2014) reported a weak but significant correlation between *Salmonella* and *E. coli* in broiler carcass rinse. In a study performed in China, the researchers tested fresh, chilled, and frozen poultry for both *Salmonella* and *E. coli* concentration in $\log_{10}$ CFU/g. The median *E. coli* population of samples negative for *Salmonella* and that of samples positive for *Salmonella* were not significantly different for chilled and frozen poultry (Li et al., 2019). In our study, similar results were observed for WCR-F samples, with the variation of *E. coli* concentration not significantly affecting the predicted probability of *Salmonella* presence.

5.4.4 *E. coli* as an indicator organism for prediction of *Listeria* presence

Geylan et al. (2008) discussed the relationship between *Listeria monocytogenes* and physiochemical factors as well as indicator organisms and found that *Enterobacteriaceae* were positively associated with *L. monocytogenes* in chicken meat. Our study showed weak but significantly positive correlation between *E. coli* population and the probability of finding *Listeria* for all five sample types, indicating the potential of using *E. coli* as an indicator of *Listeria* presence. However, a contradictory result has been reported: no significant correlation between generic *E. coli* and *L. monocytogenes* was detected in irrigation ponds (Gu et al., 2020). In general, the connection between *E. coli* and *Listeria* is biologically weak and more evidence is needed to prove the relationship.

5.5 Conclusions

This study evaluates the potential of using *E. coli* as an indicator organism for foodborne pathogens in pastured poultry farms. Logistic regression models were generated to describe the association between *E. coli* and pathogens such as *Campylobacter*, *Salmonella*, and *Listeria*. The WCR-P samples are most useful to estimate the prevalence of *Campylobacter*, *Salmonella*, and *Listeria*, while the WCR-F samples show less association between *E. coli* and the three pathogens. In general, the correlation between *E. coli* and *Listeria* is weak in feces, soil, and ceca samples. In addition, significant negative correlations were found between *E. coli* and *Campylobacter* in feces samples.

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5.7 References

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Table 5.1 Summary of sample size, type, pathogen positive rate, and *E. coli* population

Type	Sample size	Positive rate of *Campylobacter*	Positive rate of *Salmonella*	Positive rate of *Listeria*	Minimum *E. coli* population ($\log_{10}$ CFU/g)	25% quantile *E. coli* population ($\log_{10}$ CFU/g)	Median *E. coli* population ($\log_{10}$ CFU/g)	75% quantile *E. coli* population ($\log_{10}$ CFU/g)	Maximum *E. coli* population ($\log_{10}$ CFU/g)
Feces	629	61.05%	15.11%	16.22%	2.70	5.70	6.45	7.37	9.57
Soil	625	20.99%	11.48%	17.28%	0.00	2.83	4.30	5.43	8.42
WCR-P	235	15.74%	28.51%	10.21%	0.00	1.15	2.21	2.99	5.11
WCR-F	230	2.17%	17.83%	22.17%	0.00	0.70	1.36	2.44	6.90
Ceca	206	94.14%	13.11%	4.37%	0.00	4.63	5.15	5.88	8.42

Table 5.2 *p*-value of Fisher's exact independence test

	Feces	Soil	WCR-P	WCR-F	Ceca
Campylobacter vs *Salmonella*	0.0244[a]	0.0048[a]	0.0771	0.5888	0.6602
Campylobacter vs *Listeria*	0.7210	0.0007[a]	0.0181[a]	0.5892	<0.0001[a]
Salmonella vs *Listeria*	1.0000	0.7630	0.4785	0.0380[a]	0.3346

[a] *p*-value < 0.05 indicates the correlation between the pathogens

Table 5.3 Coefficients of intercept and slope by sample types for predicting *Campylobacter* presence from *E. coli*

	Campylobacter			
Sample type	Intercept	Slope	*p*-value	*p*-value of goodness of fit test
Feces	2.6601	-0.2522	0.0025[a]	0.7660
Soil	-2.2340	0.2515	<0.0001[a]	0.6592
WCR-P	-4.9740	0.3242	<0.0001[a]	0.1828
WCR-F	-4.2291	0.2276	0.4090	0.3702
Ceca	5.5128	-0.5029	0.1071	0.3405

[a] $p < 0.05$ indicates a significant correlation between *E. coli* population and *Campylobacter*

Table 5.4 Coefficients of intercept and slope by sample types for predicting *Salmonella* presence from *E. coli*

Sample type	Intercept	*Salmonella* Slope	*p*-value	*p*-value of goodness of fit test
Feces	-1.1922	-0.0551	0.5587	0.0060[b]
Soil	-2.9801	0.2449	0.0011[a]	0.1057
WCR-P	-0.2690	-0.3203	0.0157[a]	0.7053
WCR-F	-1.5958	0.0408	0.7430	0.7036
Ceca	-2.8001	0.1719	0.4282	0.3279

[a] $p < 0.05$ indicates a significant correlation between *E. coli* population and *Salmonella*

[b] $p < 0.05$ indicates the model fitting is not idea

Table 5.5 Coefficients of intercept and slope by sample types for predicting *Listeria* presence from *E. coli*

Sample type	Intercept	*Listeria* Slope	*p*-value	*p*-value of goodness of fit test
Feces	-2.6853	0.1649	0.0908	0.0773
Soil	-2.1250	0.1326	0.0372[a]	0.0949
WCR-P	-4.3025	0.8465	0.0002[a]	0.2156
WCR-F	-1.8377	0.3266	0.0039[a]	0.6054
Ceca	-4.5157	0.2678	0.4537	0.5943

[a] $p < 0.05$ indicates a significant correlation between *E. coli* population and *Listeria*

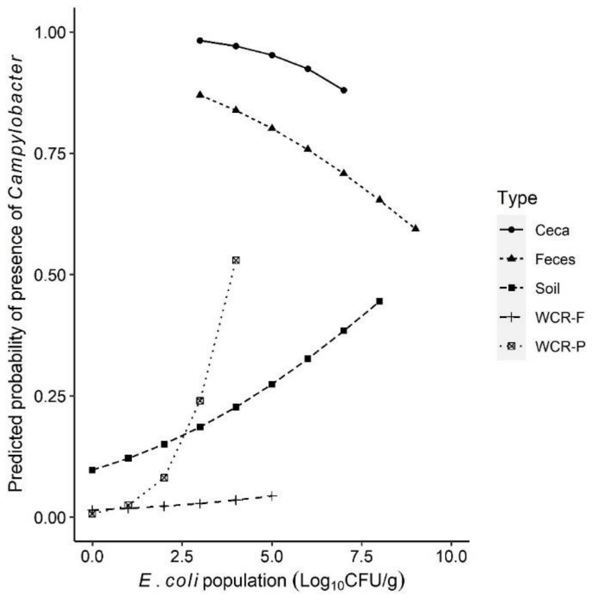

Figure 5.1 Predicted probability of *Campylobacter* over *E. coli* population

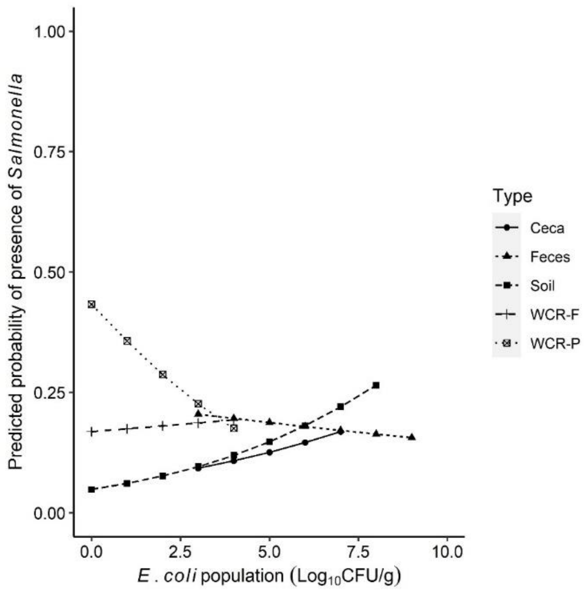

Figure 5.2 Predicted probability of *Salmonella* over *E. coli* population

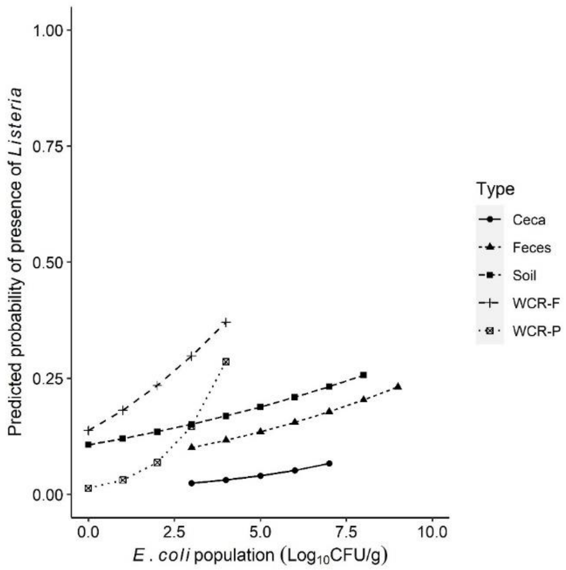

Figure 5.3 Predicted probability of *Listeria* over *E. coli* population

CHAPTER 6

THE ASSOCIATION BETWEEN PASTURED POULTRY-RELATED MICROBIOMES AND *CAMPYLOBACTER* PRESENCE

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[1] Xu, X., Rothrock Jr, M. J., Reeves, J., Kumar, G. D., & Mishra, A. (2022). Submitted to *International Journal of Food Microbiology*, 09/26/2022

Abstract

Campylobacter causes an estimated 1.5 million illness each year in the United States. Chicken is a major reservoir of *Campylobacter* and is the third most common source of contamination in *Campylobacter* outbreaks. The purpose of this study is to identify microbial taxa that differentiate *Campylobacter* positive from negative groups in pastured poultry farms. A total of 1641 samples: 543 fecal samples, 542 soil samples, 177 ceca samples, 199 whole carcass rinse during processing (WCR-P) samples, and 180 whole carcass rinse of final product after chilling and storage (WCR-F) samples were obtained from eleven pastured poultry farms. Every sample was enumerated for *Campylobacter* using the CEFEX cultural media and molecularly using microbiome relative abundances via 16S rRNA amplicon sequencing. Linear discriminant analysis (LDA) effect size (LEfSe) was used to identify differential taxa between *Campylobacter* positive and negative samples, and overall showed that *Campylobacter* positive and negative sample microbiomes differed based on sample type. For feces samples, *Bacillales* was the common taxon that was highly associated with *Campylobacter* samples by cultural and microbiome methods, while *Acidobacteria* were associated with *Campylobacter* negative samples in soil samples, according to cultural and microbiome methods. *Campylobacter* positive samples were distinguished from negative samples by the presence of *Gamma-proteobacteria* and *Bacteroidetes*. In general, the LEfSe comparison of two methods revealed that pre-harvest samples had better agreement than post-harvest samples. *Streptophyta*, *Cyanobacteria*, and Chloroplasts were significantly enriched in *Campylobacter* negative ceca, WCR-P, and WCR-F samples for post-harvest samples. This study compared the cultural and microbiome methods for detecting *Campylobacter* in pastured poultry farms. Both methods used common taxa to

represent *Campylobacter* positive or negative samples, thereby providing information for future *Campylobacter*-related taxa research.

6.1 Introduction

Campylobacter is a major cause of foodborne illnesses and outbreaks and *C. jejuni* is the most frequent agent associated with such infections. The Centers for Disease Control and Prevention (CDC) reported that among 236 foodborne *Campylobacter* outbreaks from 2010 through 2017, 41 were associated with poultry (CDC, 2017) which is the third most common cause of *Campylobacter* outbreaks. Outbreaks of *Campylobacter* associated with chicken liver were reported and *C. jejuni* was isolated using cultural methods (CDC, 2013; Scott et al., 2015). One main reason for *Campylobacter* infections is the mishandling of raw contaminated chicken meat or the consumption of undercooked chicken meat (Luber et al., 2006). Additionally, chicken has been identified as a major reservoir of *Campylobacter* for its ability to colonize the chicken gastrointestinal tract at densities as high as 10^9 CFU/g in ceca (Rosenquist et al., 2003). Feed, other farm animals, biosecurity threats (wildlife species), drinking water, soil, insects, farm equipment, employees, visitors, and farm vehicles have been identified as possible routes of *Campylobacter* transmission on poultry farms (Ghareeb et al., 2019). It is important to understand *Campylobacter* prevalence in such transmission routes and its connection to poultry-related microbiomes.

While cultural methods are typically employed to quantify *Campylobacter* in public health outbreak-related studies, *Campylobacter* can be identified and quantified in a wide range of environmental samples using molecular techniques, such as 16S rRNA amplicon sequencing-based microbiome analyses. Microbiome was first used by Whipps et al. (1988) to describe microbial associates of plants. Recently, the term microbiome has evolved and is defined as the collection of microorganisms living symbiotically on and within human, animal body, plants, or environments (Marmion et al., 2021) and the dynamics of the microbiome in response to stress and the onset of disease (Sweet and Bulling, 2017). Microbiome consists of a variety of bacteria, archaea, and eukarya; however, since only 20 – 40% of bacterial species can be cultured and identified, 16S rRNA gene amplicon sequencing provides a more complete picture of the microbial community (Gaskins et al., 2002). These communities can directly affect animal, environmental, and public health (Rothrock et al., 2019). High throughout sequencing has been widely used in outbreak investigations, surveillance, and identifying microbial community characteristics. This technology provides a way to study interactions between pathogens, commensal bacteria, and the environment. Pathogen infections in animal guts cause changes in diversity and relative abundance of bacterial taxa. In turn, the healthy gut microbiome provides protections against pathogens. Awad et al. (2016) pointed out that *C. jenuni* significantly reduced *E. coli* levels in the gut of infected broilers in both jejunum and ceca, which suggested a possibility of using *E. coli* as an important determinant of *Campylobacter* infection in chicken. Studies have shown that age (Ding et al., 2017), breed (Hou et al., 2016), diet (Qiao et al., 2018), probiotics (Chen and Yu, 2020), and antibiotics (Elokil et al., 2020) can all significantly influence the microbiome of chickens. In addition, the microbiome of farm litter management (Dumas et al., 2011; Torok et al., 2009), farm soil (Crippen et al., 2019; Rothrock and Locatelli, 2019), farm dust (Luiken et al., 2020; O'Brien et al., 2016), and whole carcass rinse during processing (Handley et al., 2018; Kim et al., 2019) were studied to understand the impact of farm environment, farm management practices, and processing practices on poultry microbiomes.

While the conventional U.S. poultry industry continues to grow, there is an increasing demand for alternatives, such as pastured products. Pastured poultry is one alternative production system that requires flocks to have continual access to fresh pasture and the outdoor environment on a daily basis (Sossidou et al., 2019). Meanwhile, surveys have shown that consumers are supportive of all-natural products (Dailey et al., 2017). In addition, the public has shown a willingness to pay more for pastured products that are locally grown. Previous studies have investigated the impact of farm environment on pasture-related microbiomes (Rothrock and Locatelli, 2019), the effect of soy-based diet on pastured poultry (Lourenco et al., 2019a; Lourenco et al., 2019b), and the ecology of pasture-related microbiomes (Rothrock et al., 2019). However, there is limited research available discussing pastured poultry-related microbiome and the resulting foodborne pathogens prevalence. Our goal is to investigate the connection between microbial communities and *Campylobacter* presence in pastured poultry farms. Moreover, we will compare cultural-identified *Campylobacter* prevalence and microbiome-identified *Campylobacter* prevalence to determine the potential for using microbiome relative abundance data in foodborne pathogen outbreak investigations.

6.2 Materials and methods

Environmental samples and chicken carcass rinse samples were collected from 42 flocks in 11 pastured poultry farms in the southeastern U.S. from March 2014 to November 2017. In the study, all broilers reared in the 11 farms were pastured chickens. Different types of samples were collected from each flock: (i) feces, (ii) pasture soil, (iii) ceca samples, (iv) whole carcass rinse directly after processing (WCR-P), and (v) final product whole carcass rinse after chilling and storage time (WCR-F) collected during processing from each farm. In total, 1641 samples were included including 543 fecal samples, 542 soil samples, 177 ceca samples,199 WCR-P samples, and 180 WCR-F samples.

6.2.1 Sample preparation

Feces and soil samples were taken three times throughout a flock's lifecycle: (i) within a few days of being placed in the pasture (Start), (ii) halfway through their time on pasture (Middle), and (iii) on the day the flock was processed (End). On each sampling day, the pasture area was divided into five separate sections. Then five subsamples in each section were pooled into a single sample for each section (a total of five soil samples and five feces' samples were collected on each sampling day). Soil samples were collected from the surface (0–7 cm) with sterile scoops, and feces samples were collected from fresh droppings on the soil surface. Gloves and scoops were changed between sample types and between sampling areas. During the on-farm processing day, ceca and carcass rinse samples were collected. Upon evisceration, cecal sacs from five carcasses were removed and placed into a single sampling bag to create a pooled sample. A total of five pooled samples (n = 5) was created. Gloves and scissors were changed between each pooled sample. Prior to packaging and storage of the carcasses for the consumer, each of the 25 carcasses were placed in sterile plastic bags, rinsed with 100 ml of 10 mM phosphate-buffered saline (PBS) and vigorously shaken for 1min. Whole carcass rinses (WCR) from five carcasses were pooled together in a filtered stomacher bag creating five pooled samples (n = 25).

6.2.2 *Campylobacter* enumeration

Samples were prepared as previously described by Rothrock and Locatelli (2019). Briefly, 3 g from each subsample were combined in a filtered stomacher bag (Seward Laboratory Systems, Inc., Davie, FL) and diluted 1:3 with 10 mM phosphate buffered saline (PBS) and homogenized for 1 min. Next, 100 μL of homogenized sample was plated onto Campy-Cefex agar (Neogen, Lansing MI) and incubated at 42 ± 1 ℃ in microaerophilic conditions (85% N_2, 10% CO_2, 5% O_2) for 36-48 h (Stern et al., 1992). Putative *Campylobacter* colonies were enumerated for each plate, and up to 5 suspected colonies were transferred to Brucella agar (Neogen, Lansing MI) supplemented with 10% lysed horse blood (Lampire Biological Laboratories, Pipersville PA) for confirmation and incubated. For model development purposes, samples were classified as positive if countable colonies were found during Campy-Cefex plating. This is referred to hereafter as classification by the cultural method, as shown in the top row of Table 6.2.

6.2.3 DNA extraction and quantification

DNA extractions were performed on 0.33 g of feces, 0.33 g of soil, and 0.5ml of cecal homogenate and 0.5ml of WCR. DNA was extracted from samples according to a semi-automated hybrid DNA extraction protocol previously described (Rothrock et al., 2014). This method was a combination of a mechanical method using the FastDNA Spin Kit for Feces (MP Biomedicals, Solon, OH, USA) and an enzymatic method based on the QIAamp DNA Stool Mini Kit (QIAGEN, Valencia, CA). DNA purification was performed using the DNA Stool—Human Stool—Pathogen Detection Protocol of the QIAcube Robotic Workstation. After purification, the DNA concentration in each sample was determined spectrophotometrically using the Take3 plate in conjunction with the Synergy H4multimode plate reader (BioTek,Winooski, VT). For this classification method, known as the microbiome method, and displayed numerically in the second row of Table 6.2, samples were classified as *Campylobacter* positive if the relative abundance of taxon *Campylobacter* was greater than zero and *Campylobacter* negative if the relative abundance was zero.

6.2.4 16S rRNA gene sequencing

Library construction and sequencing were performed by the Earth Microbiome Project Laboratory at the U.S. Department of Energy, Argonne National Laboratory (Argonne, IL, USA). Briefly, the hypervariable V4 domain of bacterial 16S rRNA gene was amplified using the F515 (5′ -CACGGTCGKCGG CGCCATT-3′) and R806 (5′ -GGACTACHVGGGTWTCT AAT-3′) primer set with each primer containing Illumina adapter regions (Illumina, Inc., San Diego, CA, USA) and the reverse primer containing the Golay barcodes to facilitate multiplexing (Caporaso et al., 2011)The initial demultiplexing and quality filtering steps were performed using the QIIME pipeline v1.9.1 (Caporaso et al., 2010b) . The 16S rRNA gene amplicon pool was demultiplexed by internal barcodes to identify individual samples and primers were removed. Sequences were then chimera-checked against the Greengenes 13_8 database (DeSantis et al., 2006) and clustered into Operational Taxonomic Units (OTUs) according to a similarity set at 97%. The open-reference OTU picking protocol was used. A representative sequence for each OTU was selected and used for taxonomic assignment using UCLUST and the Greengenes 13_8 database. Sequences were aligned using PyNAST (Caporaso et al., 2010a), and filtered. A phylogenetic tree was subsequently produced, and singleton OTUs and OTUs whose representative sequences could not be aligned with PyNAST were removed.

6.2.5 Statistical analysis

For each sample, the OTU counts were normalized so that the sum over all taxa is one. The relative abundance values were used in the following analyses. Altogether, 1824 unique taxa were identified. To avoid the issue of sparsity with many zeros, the mean relative abundance was calculated for each OTU and the mean OTU greater than the 75 percentile was kept. That is, this filtration process retained the 456 most abundant OTUs (within each of the five sample types) for performing the analyses. After the filtration, 99.9% of the relative abundance was remained. The analyses were conducted at genus level.

Campylobacter positive and negative samples were identified by cultural method (section 2.2) and microbiome method (section 6.2.3). Linear discriminant analysis effect size (LEfSe) (http://huttenhower.sph.harvard.edu/galaxy/) was conducted to identify genomic features characterizing the difference among groups (Segata et al., 2011). The linear discriminant analysis (LDA) score was calculated, and a taxonomic cladogram was constructed to visualize the differential taxa. The LDA score greater than four was used as threshold for the LEfSe analysis. The LEfSe algorithm uses Kruskal-Wallis test ($\alpha = 0.05$) to check difference between classes (*Campylobacter* positive and negative groups). To compare cultural method and microbiome methods, sensitivity, specificity, and accuracy were calculated (Equation 1, 2, and 3). In this calculation, cultural method was set as reference method, and we are looking at agreement in a 2-by-2 table for each of the sample types.

$$Sensitivity = \frac{TP}{TP+FN} \tag{1}$$

$$Specificity = \frac{TN}{TN+FP} \tag{2}$$

$$Accuracy = \frac{TP+TN}{TP+FN+TN+FP} \tag{3}$$

Where TP is true positive, FN is false negative, TN is true negative, and FP is false positive.

6.3 Results and Discussion

6.3.1 *Campylobacter* prevalence (culture-based) or relative abundance (microbiome-based)

Figure 6.1 displays the bar plot of relative abundance by sample types at phylum level. *Firmicutes* and *Proteobacteria* were the top two phylum across all sample types. This result is in agreement with Videnska et al. (2014), Lee et al. (2017), Handley et al. (2018), and Kim et al.

(2019) in feces, ceca, WCR-P, and WCR-F samples. The top five most frequent taxa of different sample types are shown in Table 6.1. *Lactobacillus* was the most frequent taxon in feces and soil samples with a relative abundance of 0.526 and 0.065, respectively. For ceca samples, *Ruminococcaceae* was the taxon with highest relative abundance (0.148). The relative abundances of *Acinetobacter* were 0.173 and 0.165, which were the most frequent microbial taxa in WCR-P and WCR-F samples, respectively. Overall, the top five OTUs comprised 72% of the relative abundance for feces samples. For soil samples, the top five taxa comprised less than 20%, while for the other three, the top five OTUs comprised between 44% to 49% of the relative abundance. Acinetobacter was the only taxa in the five most abundant taxa in every sample type.

Table 6.2 shows *Campylobacter* prevalence and sample size by sample types. *Campylobacter* prevalence showed differences within sample types by cultural and microbiome methods. For example, 28.8% of *Campylobacter* positive samples was identified in soil samples by cultural method. However, by microbiome method, the *Campylobacter* prevalence was 62.9%. *Campylobacter* was very prevalent in ceca and feces samples (94.4% and 74.2%) by cultural method where the prevalence was 60.5% and 69.2% by microbiome method. Other studies have reported high *Campylobacter* prevalence in ceca and feces samples using cultural method (Stern and Robach, 2003; Tang et al., 2020; Tangkham et al., 2016; Xu et al., 2021). Because the microbiome is based on relative abundance (rather than true abundance), larger bacterial populations may result in a high number of false positives. For example, if one can only detect taxa greater than 0.001% of the community and the community density is 10^{10} cells/g, anything less than 10^{5} cells/g will be missed (but these taxa could be observed culturally since growth of *Campylobacter* was selected only). In WCR-P and WCR-F samples, lower *Campylobacter* prevalence were detected (17.6% and 22.2%, respectively) by cultural method compared to microbiome method (47.7% and 50.6%, respectively). From Table 6.2, we can see that the microbiome method identified a higher proportion of positive sample than did the cultural method for the soil, WCR-P, and WCR-F sample types, while the opposite was true for the feces and ceca sample types. This could be due to *Campylobacter* entering a viable but non-culturable (VBNC) state upon exposure to various stress, including low temperature, oxygen, acid treatment, and salt treatment (Lv et al., 2019). In addition, the pathogen lacks many stress-response mechanisms found in other Gram negative bacteria, making it sensitive to stress conditions and easy to enter VBNC state (Magajna and Schraft, 2015). This could explain the low prevalence of *Campylobacter* in soil, WCR-P, and WCR-F samples, particularly in WCR samples where *Campylobacter* may have been stressed during processing and storage. Table 6.3 summarizes sensitivity, specificity, and accuracy by samples using the cultural data as the reference and the microbiome method as the testing method. Sensitivity is the ability of a method to correctly classify true positive where specificity is the ability of a method to correctly classify true negative (Parikh et al., 2008). Sensitivity also measures the rate of a method to incorrectly classify negative samples as positive samples (false negative). Meanwhile, specificity is the rate at which a method incorrectly classifies positive samples as negative samples (false positive). In our circumstances, a higher specificity value is more important than a higher sensitivity value. This is because false positive samples increase the risks of food safety.

The sensitivities of feces and soil samples were 0.757 and 0.833, indicating that microbiome method correctly classified 75.7% and 83.3% *Campylobacter* positive feces and soil samples respectively, and the number of false negative samples was low. This suggests that microbiome relative abundance data can correctly identify *Campylobacter* positive samples preharvest (feces and soil) samples; however, the specificities of feces and soil samples were

0.493 and 0.453, respectively, indicating that there is a high chance of microbiome method to incorrectly identify *Campylobacter* positive samples as negative samples. The low specificity indicates that there is an increased likelihood of misclassifying *Campylobacter* positive samples as negative samples. Similarly, for WCR-P and WCR-F samples, the specificities were 0.494 and 0.489, respectively, indicating that the microbiome method correctly identified 49.4% and 48.9% of true negative samples. Accuracy is calculated by dividing the number of measurements correctly made (both true positive and true negative) to the total number of measurements. Higher accuracy means better performance of the testing method (microbiome method) compared to the reference method (cultural method). The highest accuracy was achieved in feces samples and the lowest accuracy was in WCR-P samples. In summary, microbiome method showed relative low accuracy in terms of cultural methods. Furthermore, the likelihood of incorrectly classifying *Campylobacter* positive samples as negative samples was high by microbiome method.

6.3.2 LEfSe analysis for preharvest samples

Not only is it important to compare the overall efficacy of using cultural versus microbiome-based quantification of *Campylobacter* in this study, but we also wanted to know what other taxa in the microbiome data correlated with *Campylobacter* positive and negative samples. To accomplish this, LEfSe analyses were used to determine the microbiome taxa that were significantly related the *Campylobacter* status of a sample, whether assessed culturally or via microbiome data. The linear discriminant analysis (LDA) effect size (LEfSe) method is used to detect operational taxonomic units (OTUs) that are most likely to explain the differences between classes by coupling standard tests for statistical significance with additional tests encoding biological consistency and effect relevance (Segata et al., 2011). This method has been used to detect the difference between production systems (Pandit et al., 2018), growth performance (Zhang et al., 2022), microbial contamination in slaughter house (Song et al., 2021), and other studies (Chen et al., 2019; Cui et al., 2021; Wang et al., 2021).

6.3.2.1 Feces

Figure 6.2 shows the comparison of the fecal LEfSe results. Taxa such as *Proteobacteria*, *Gammaproteobacteria*, and *Bacillales* were significantly abundant in *Campylobacter* negative samples by cultural method, while *Firmicutes*, *Bacilli*, *Lactobacillales*, *Lactobacillaceae*, and *Lactobacillus* were significantly abundant in *Campylobacter* positive samples (Fig. 6.2A). When assessing the microbiome-based *Campylobacter* status (Fig. 6.2B), there were no taxa significantly enriched in the positive samples, while only 3 taxa (*Enterococcaceae*, *Enterococcus*, and *Bacillales*) were significantly abundant in the negative samples.

Previous studies have documented that *Firmicutes*, in particular *Lactobacillus* were dominant in the gastrointestinal microbiome of broiler chickens (Danzeisen et al., 2011; Qu et al., 2008; Stanley et al., 2013), so their inclusion as important taxa in this study was not surprising. Dicksved et al. (2014) reported that *Bacteroides*, *Escherichia*, *Phascolarctobacterium*, and *Streptococcus* were associated with *Campylobacter* positive chicken feces samples. This is in agreement with the current study, considering *Streptococcus* belongs to order *Lactobacillales* and phylum *Firmicutes*, two of the taxa significantly abundant in the cultural *Campylobacter* positive samples (Fig 6.1A). Moreover, *Firmicutes* were lower and *Proteobacteria* were higher in abundance in *Campylobacter* positive samples compared to negative samples (Sofka et al., 2015) where our results suggest opposite outcomes. *Bacillales* was the common taxon significantly abundant in *Campylobacter* negative samples using both the cultural and microbiome data. Patuzzi et al. (2021) found lower *Bacillales* abundance in

Campylobacter positive samples where our results suggest higher *Bacillales* levels in *Campylobacter* negative samples. The high level of *Bacillales* in *Campylobacter* negative samples may suggest that genera including *Bacillus*, *Listeria*, and *Staphylococcus* may compete with *Campylobacter*. In total, these suggest that *Firmicutes* and lactic acid bacteria may have important interactions with *Campylobacter* in the broiler gut, and more research is needed to identify the specific protagonistic or antagonistic genera and species as well as the mechanisms used to enhance or inhibit *Campylobacter* colonization.

6.3.2.2 Soil samples

For soil samples, *Acidobacteria* was significantly associated with *Campylobacter* negative samples using either the cultural (Fig 6.3A) or microbiome (Fig 6.3B) data, with taxa related to *Bacillales* and *Actinobacteria* also significantly abundant using the microbiome data. For the *Campylobacter* positive samples, taxa related *Gammaproteobacteria* and *Bacteriodetes* were significantly enhanced using either the cultural (Fig. 6.2A) or microbiome (Fig. 6.2B) data. Five other taxa (*Pseudomonadales*, *Enterobacteriaceae*, *Enterobacteriales*, *Lactobacillales*, *Proteobacteria*) were also found to be significantly abundant in *Campylobacter* positive samples using the microbiome data.

Acidobacteria was the common taxon found by cultural and microbiome method in soil *Campylobacter* negative samples. Previous work has shown that the abundance of *Acidobacteria* may decrease under precipitation and the presence of *Proteobacteria* (Yang et al., 2019). This could explain the high abundance of *Acidobacteria* in *Campylobacter* negative samples with no dominant *Proteobacteria*. Also, the high levels of *Proteobacteria* in *Campylobacter* positive samples resulted in no significant presence of *Acidobacteria*. For *Campylobacter* positive samples, *Gammaproteobacteria* and *Bacteroidetes* were the common taxa based on both cultural and microbiome data. *Bacteroidetes* are major members in gastrointestinal tract and are regarded as specialists for the degradation of high molecular weight organic matter such as proteins and carbohydrates (Thomas et al., 2011). This may assist the growth of *Campylobacter* in gastrointestinal tract and then they may pass to soil through feces.

6.3.3 LEfSe analysis for postharvest samples

Unlike the preharvest results (feces, soil) described above, many taxa were found to be significantly associated with *Campylobacter* positive or negative postharvest samples (ceca, WCR-P, and WCR-F in Fig. 6.4, 6.5, and 6.6, respectively). Therefore, only those taxa demonstrating the greatest LDA scores will be discussed in detail below.

6.3.3.1 Ceca samples

Figure 6.4 displays LEfSe results by cultural and microbiome method for ceca samples. When detecting *Campylobacter* culturally (Fig. 6.4A), taxa related to *Clostridiales*, *Clostridia*, *Firmicutes,* and *Ruminococcaceae* were significantly enriched (LDA score > 4.8) in *Campylobacter* negative samples, while a taxon related to *Proteobacteria* was significantly enriched in *Campylobacter* positive samples. This is in agreement with previous work that has shown that *Campylobacter* negative samples have higher amounts of *Firmicutes*, whereas a higher proportion of *Proteobacteria* was found in *Campylobacter* positive samples (Sofka et al., 2015). When detecting *Campylobacter* molecularly via microbiome analysis (Fig. 6.4B), taxa related to Chloroplasts, *Streptophyta*, and *Proteobacteria* were enriched in *Campylobacter* negative samples, while taxa related to *Clostridia*, *Clostridiales*, and *Firmicutes* were enriched in the *Campylobacter* positive cecal samples. This is in line with with Sakaridis et al. (2018) that the percentages of *Clostridiales* increased as *Campylobacter* concentration increased. Chloroplasts may be originated from plant (feed) (Rychlik, 2020) and its reads have been utilized as a proxy for abundance of plant dietary content (Clayton et al., 2016; David et al., 2014). Saxena et al. (2016) reported that *Streptophyta* was a scarce taxon in ceca samples. Azcarate-Peril et al. (2018) identified *Streptophyta* in ceca and originated it from feed. *Streptophyta* was positively correlated with a variety of major metabolites, however, little information is known about this taxon (Wei et al., 2021). The variation and even contradictory results obtained from different studies may be due to sampling method, library, production type (traditional or pastured), or environmental conditions, so these variables need to be considered when comparing results and implementing microbiome data into outbreak studies.

Our results showed that *Campylobacter* positive samples were high in abundance of *Firmicutes* (*Clostridia, Clostridiales*, and *Ruminococcaceae*). This is line with Awad et al. (2016), that showed *Campylobacter* colonization shifted the cecal microbiome to an enrichment of *Firmicutes* and concomitant reduction of *Proteobacteria*. *Firmicutes* is known to be involved in the degradation function of complex carbohydrates and in the production of short-chain fatty acids (SCFAs) (Thibodeau et al., 2015). Since *Campylobacter* requires high SCFAs as an energy source to grow, it may explain the dominance of *Firmicutes* in *Campylobacter* positive samples. Connerton et al. (2018) identified *Clostridiales* one of the highly abundant taxa in *Campylobacter* colonized ceca samples, which agrees with our data as well as Awad et al. (2016) and Kaakoush et al. (2014). It was postulated that *Campylobacter* acts as a hydrogen sink that would improve growth of clostridial microorganisms. Then, the growth of clostridial organisms produce organic acids through fermentation, which can be used by *Campylobacter* as an energy source (Thibodeau et al., 2015).

6.3.3.2 WCR samples

In whole carcass rinse samples collected after processing (WCR-P; Fig. 6.5), *Proteobacteria*, *Gammaproteobacteria*, and *Pseudomonadales* were the top three taxa significantly associated with *Campylobacter* negative samples when assessed culturally, while no taxa were specifically enriched in *Campylobacter* positive samples (Fig. 6.5A). When assessing *Campylobacter* molecularly using microbiome (Fig. 6.5B), *Campylobacter* negative samples were significantly enriched in taxa related to Chloroplast, *Streptophyta*, and *Proteobacteria*, while *Campylobacter* positive samples were enriched in taxa related to *Clostridia*, *Clostridiales*, and *Firmicutes*.

In the final product whole carcass rinses samples (WCR-F; Fig. 6.6), *Flavobacteria*, *Flavobacterium*, and *Flavobacteriaceae* were the most enriched taxa in *Campylobacter* positive samples when assessed culturally, while no taxa were found to be enriched in the *Campylobacter* negative WCR-F samples (Fig. 6.6A). *Flavobacterium* is a Gram-negative bacteria have been associated with meat spoilage (Blickstad et al., 1981) and Song et al. (2021) found *Flavobacterium* one of the dominant taxa in chicken carcasses produced during warmer growing seasons. The high abundance of *Flavobacteria* in *Campylobacter* positive WCR-F samples suggested the possible correlation with *Campylobacter* colonization and potential spoilage issues. Using the microbiome detection of *Campylobacter* (Fig 6.6B), taxa related to Chloroplast, *Streptophyta,* and *Cyanobacteria* were once again enriched in *Campylobacter* negative samples, while taxa related to *Proteobacteria*, *Gammaproteobacteria*, and *Enterobacteriaceae* significantly enriched in the *Campylobacter* positive WCR-F samples. Rothrock et al. (2019) analyzed whole carcass rinse for final product and found that *Firmicutes* and *Proteobacteria* accounted for >85% of all OTUs, with *Proteobacteria* significantly more abundant in WCR samples than in feces and ceca samples.

There were several other taxa that were significantly enriched in the *Campylobacter* positive WCR-F samples and considering these are the final product samples that is directly provided to the consumer (meaning the greatest impact on public health), these associations should be highlighted. *Campylobacter*, *Campylobacteraceae*, and *Campylobacterales* were consistently abundant in in *Campylobacter* positive WCR-F samples. *Campylobacteraceae* was identified by LEfSe in *Campylobacter* positive samples from Italian poultry farms (Di Marcantonio et al., 2022), which is line with our results that *Campylobacter*, *Campylobacteraceae,* and *Campylobacterales* are more abundant. *Acinetobacter*, belonging to the *Moraxellaceae* family, was more abundant in *Campylobacter* positive WCR-F samples. It has been detected in chicken processing environment (Kanaan et al., 2020; Wu et al., 2017) as well as chicken carcasses and chicken breast and fillets (Dourou et al., 2021; Savin et al., 2020; Wang et al., 2017). *Pseudomonadales* was consistently abundant in *Campylobacter* positive WCR-F samples where *Pseudomonas* has been recognized as a predominant meat spoiler (Doulgeraki et al., 2012; Nychas et al., 2008; Remenant et al., 2015). Combined with high levels of *Acinetobacter*, these data indicated that a high abundance of spoilage microorganisms may indicate the presence of *Campylobacter*.

Interestingly, taxa related to *Streptophyta*, *Cyanobacteria*, and Chloroplasts were greatly enriched in all three *Campylobacter* negative postharvest sample types (ceca, WCR-P, WCR-F) based on the microbiome data (Fig 6.4B, 6.5B, 6.6B, respectively). These three taxa are associated with photosynthetic members of the farm microbiome (Braissant et al., 2007; Graham et al., 2014), and considering the broilers included in this study were all pasture-raised, may be a result of the ingestion of foraged plant material. Cyanobacterial species known to produce heterocytes that are involved in N fixation (Knack et al., 2015). Given their dominance in *Campylobacter* negative postharvest samples, more research should focus on these possible antagonistic relationships and what role foraging may play in potentially reducing the abundance of *Campylobacter* within the pastured broiler gut.

6.4 Conclusions

In conclusion, the present study identifies taxa that differentiate between *Campylobacter* positive and negative samples by sample types and by different methods. For feces samples, *Bacillales* was the common taxon that was highly abundant in *Campylobacter* negative samples by both methods. In soil samples, *Acidobacteria* was high in abundance in *Campylobacter* negative samples by cultural and microbiome method. *Gamma-proteobacteria* and *Bacteroidetes* were also common features that differentiate *Campylobacter* positive samples from negative samples. In general, the LEfSe comparison of two methods showed better agreement for pre-harvest samples than for post-harvest samples. For post-harvest samples, *Streptophyta*, *Cyanobacteria*, and Chloroplasts were greatly enriched in *Campylobacter* negative ceca, WCR-P, and WCR-F samples. Furthermore, cultural method showed higher *Campylobacter* prevalence in feces and ceca samples where microbiome method showed lower prevalence. Conversely, lower *Campylobacter* prevalence was detected by cultural method but was higher in microbiome method for soil, WCR-P, and WCR-F samples. When setting cultural method as the reference method, microbiome method showed relatively low sensitivity, specificity, and accuracy for all five sample types. This study provided the comparison of cultural and microbiome method to investigating *Campylobacter* prevalence in pastured poultry farms. Taxa significantly associated with *Campylobacter* positive or negative samples were identified, providing data for future *Campylobacter*-related taxa research.

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Table 6.1 The top five most frequent taxa (relative abundance) by sample types

Taxa	Feces	Soil	Ceca	WCR-P	WCR-F
#1	*Lactobacillus* (0.526)	*Lactobacillus* (0.065)	*Ruminococcaceae* (0.148)	*Acinetobacter* (0.173)	*Acinetobacter* (0.165)
#2	*Acinetobacter* (0.070)	*Acinetobacter* (0.048)	*Clostridiales* (0.119)	*Streptophyta* (0.168)	*Streptophyta* (0.134)
#3	*Enterobacteriaceae* (0.048)	*Bacillus* (0.034)	*Streptophyta* (0.069)	*Lactobacillus* (0.067)	*Lactobacillus* (0.059)
#4	*Streptococcus* (0.041)	*Chitinophagaceae* (0.027)	*Acinetobacter* (0.053)	*Lactococcus* (0.041)	*Enterobacteriaceae* (0.058)
#5	*Enterococcus* (0.033)	*Enterobacteriaceae* (0.023)	*Oscillospira* (0.048)	*Enterobacteriaceae* (0.039)	*Lactococcus* (0.053)

Table 6.2 *Campylobacter* prevalence for cultural and microbiome methods by sample types

	Feces	Soil	Ceca	WCR-P	WCR-F
Cultural method	74.2%	28.8%	94.4%	17.6%	2.2%
Microbiome method	69.2%	62.9%	60.5%	47.7%	50.6%
Sample size	543	542	177	199	180

Table 6.3 Sensitivity, specificity, and accuracy based on sample types

	Feces	Soil	Ceca	WCR-P	WCR-F
Sensitivity	0.757	0.833	0.593	0.343	0.250
Specificity	0.493	0.453	0.200	0.494	0.489
Accuracy	0.689	0.563	0.571	0.467	0.483

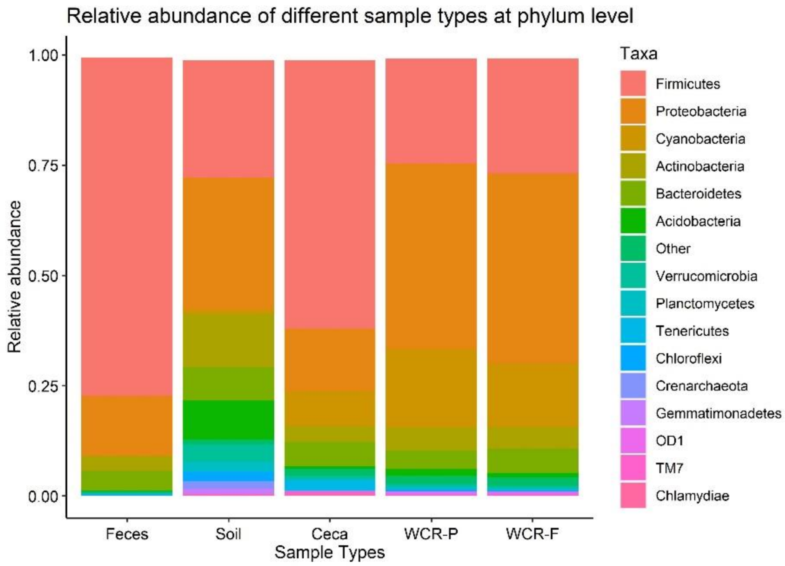

Figure 6.1 Bar plot of relative abundance by sample types at phylum level

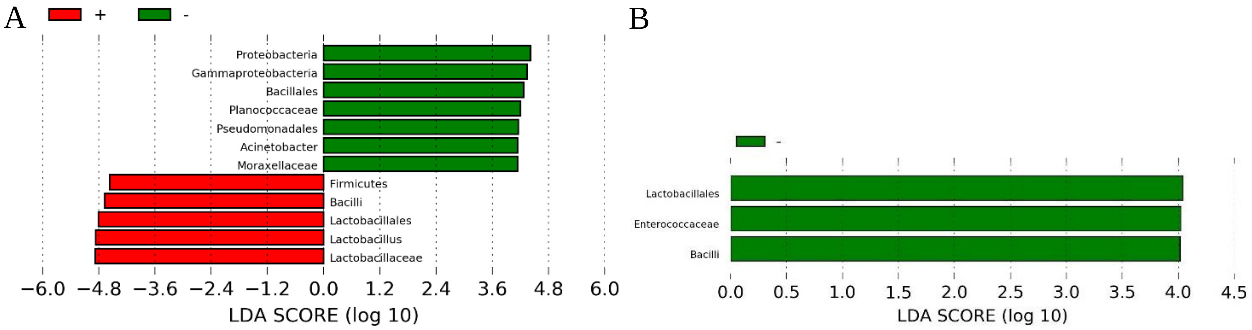

Figure 6.2 Fecal LEfSe comparison between *Campylobacter* positive (red) and negative (green) samples as assessed by the cultural method (A) or the microbiome method (B)

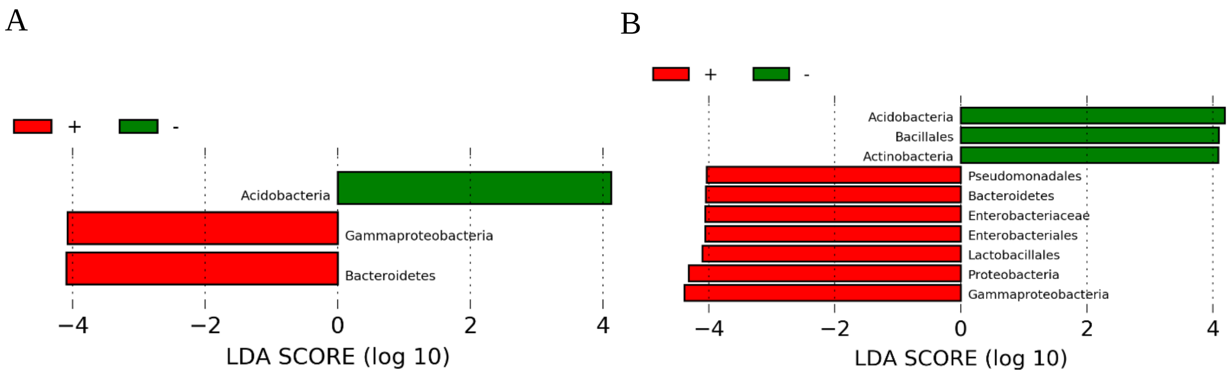

Figure 6.3 Soil LEfSe comparison between *Campylobacter* positive (red) and negative (green) samples as assessed by the cultural method (A) or the microbiome method (B)

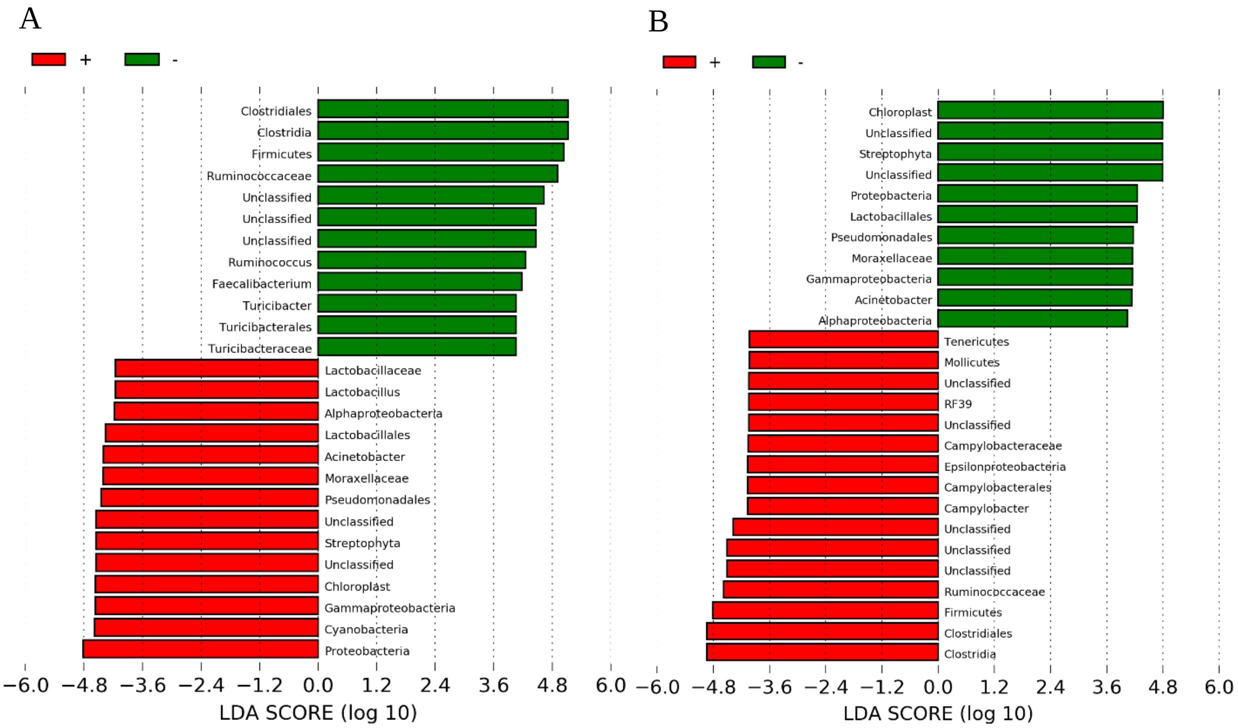

Figure 6.4 Cecal LEfSe comparison between *Campylobacter* positive (red) and negative (green) samples as assessed by the cultural method (A) or the microbiome method (B)

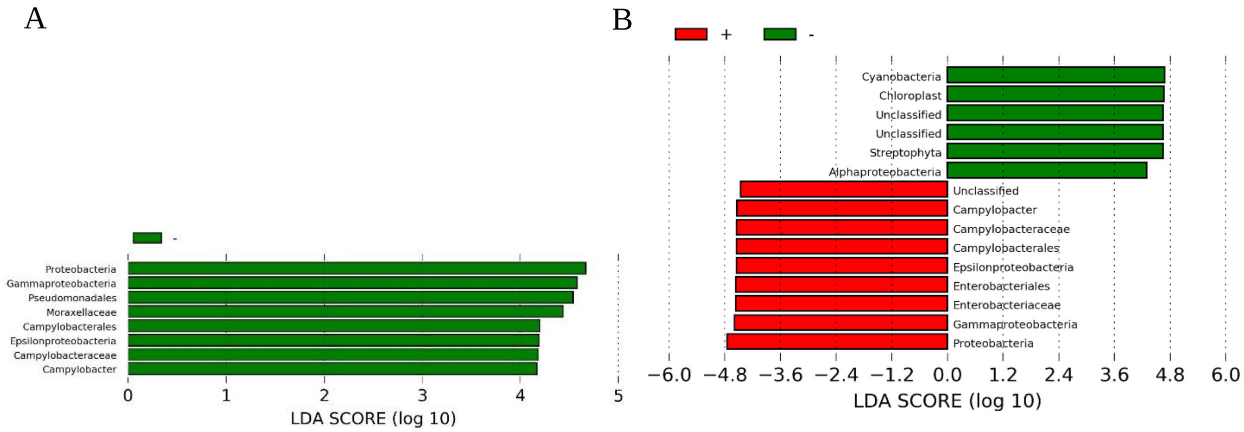

Figure 6.5 Whole carcass rinse after processing (WCR-P) LEfSe comparison between *Campylobacter* positive (red) and negative (green) samples as assessed by the cultural method (A) or the microbiome method (B).

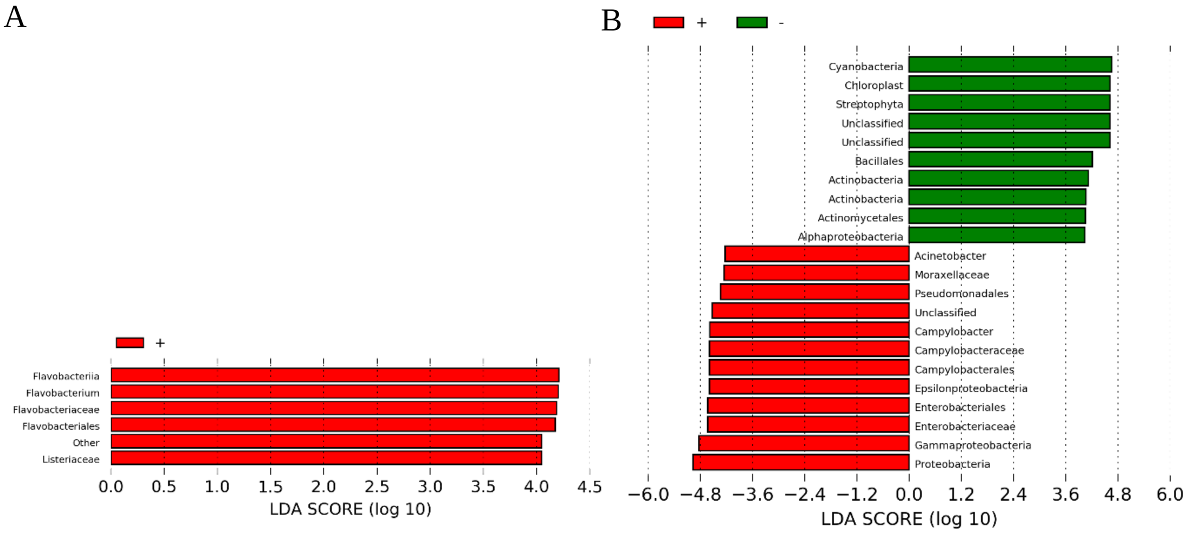

Figure 6.6 Final product whole carcass rinse (WCR-F) LEfSe comparison between *Campylobacter* positive (red) and negative (green) samples as assessed by the cultural method (A) or the microbiome method (B)

CHAPTER 7

COMPARING PASTURED POULTRY-RELATED MICROBIOMES AND CULTURAL METHOD FOR DETECTING S*ALMONELLA* PRESENCE

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[1] Xu, X., Rothrock Jr, M. J., Reeves, J., Kumar, G. D., & Mishra, A. (2022). Submitted to *Food Research International*, 10/18/2022

Abstract

Salmonella is responsible for one million foodborne illnesses in the United States each year. *Salmonella* infection can alter microbiome composition in chickens. The purpose of this study is to investigate the connection between pastured poultry-related microbiome and *Salmonella* presence. In total, 1461 samples were obtained from eleven pastured poultry farms including 543 fecal samples, 542 soil samples, 177 ceca samples, 199 whole carcass rinse during processing (WCR-P) samples, and 180 whole carcass rinse of final product after chilling and storage (WCR-F) samples. Each sample was assessed for *Salmonella* using brilliant green sulfa with novobiocin (BGS) agar and xylose lysine tergitol-4 (XLT-4) agar and molecularly using microbiome relative abundances via 16S rRNA amplicon sequencing. Results showed that the cultural method exhibited lower *Salmonella* prevalence than microbiome method in all sample types. While this comparison is important, the advantage of using microbiome-based detection is that it allows assessment of other microbial taxa that are possibly correlated to *Salmonella* throughout the farm-to-fork continuum. Linear discriminant analysis (LDA) effect size (LEfSe) was used to identify taxa significantly enriched in *Salmonella* positive samples or negative samples. In terms of pre-harvest samples, *Planococcaceae* and *Bacillales* were identified as the common taxa enriched in *Salmonella* positive fecal samples whether assessed by cultural or microbiome methods, while taxa related to *Gamma-proteobacteria* were enriched in soil samples. *Pseudomonadales* and *Gamma-proteobacteria* taxa were enriched in *Salmonella* positive WCR-P samples, while taxa related to *Streptophyta*, *Cyanobacteria*, and Chloroplasts were greatly enriched in *Salmonella* negative WCR-P samples. In general, pre-harvest samples had better agreement than post-harvest samples, according to the LEfSe comparison of two *Salmonella* detection methods. These results highlight the potential of using microbiome

analyses for foodborne pathogen detection in farm-to-fork studies, as well as identifying potential correlations between bacterial taxa and *Salmonella* at different stages of production.

7.1. Introduction

Bacteria enter the digestive system through ingestion. The indigenous bacteria start to establish complex commensal relationships within the host environment (mucosa or digestive tract), to form microbiome (Chambers and Gong, 2011). The host's immunological response, nutrient uptake, and generation of metabolites necessary for the host are significantly shaped by the gastrointestinal tract (Gaboriau-Routhiau et al., 2009; Hill et al., 2010; Ismail et al., 2009; Lillehoj, 1993). In turn, diet, feed additives, antibiotic, probiotic, breed, and age are reported to impact gut microbial communities and the gut composition (Ding et al., 2016; Elokil et al., 2020; Hou et al., 2016; Lourenco et al., 2019a; Oakley et al., 2018; Thibodeau et al., 2015; Zhu et al., 2020). Among these activities, the ability of gut microbiome to resist pathogen colonization is of special interest.

Salmonella is one of the most important pathogenic bacteria causing foodborne illnesses and outbreaks. *Salmonella* infections are the second most common cause of foodborne illnesses resulting in 20,000 hospitalizations and 400 deaths in United States (Scallan et al., 2011). In *Salmonella* outbreaks related to raw chicken or chicken product, cultural methods are commonly used for investigation and confirmation (CDC, 2018, 2019). In chickens, *Salmonella* strains can cause a fatal systemic infection, but more usually they develop into a long-lasting asymptomatic carrier state (Velge et al., 2005). Chickens that carry *Salmonella* are a severe threat to both animal and human health. Indeed, these chickens that excrete high levels of *Salmonella* in their feces can contaminate the housing, soil, and carcasses during slaughter (Kempf et al., 2020).

While cultural methods are commonly used to quantify *Salmonella* in public health outbreak-related studies, *Salmonella* and microbial community members can be identified in a variety of environmental samples using molecular techniques such as 16S rRNA amplicon sequencing-based microbiome analyses. Previous studies have shown that poultry-related microbiomes are dependent upon the types of samples that were collected along the farm-to-fork continuum. For example, the fecal microbial community is primarily composed of *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes* (Lourenco et al., 2019b), while the top phyla in cecal microbiomes are ordered differently (*Firmicutes* and *Bacteroidetes* followed by *Actinobacteria* and *Proteobacteria*) (Rychlik, 2020). *Firmicutes* and *Bacteroidetes* typically represent around 45% of the overall microbiota in the cecal microbiome of healthy adult chickens (Rychlik, 2020). *Actinobacteria* and *Proteobacteria* typically make up 2% to 3% of the overall microbiota, yet studies utilizing 16S rRNA sequencing may underestimate the amount of *Actinobacteria* because they only have one to five copies of the 16S rRNA gene (Candela et al., 2004). Additionally, dysbiosis can occur by the presence of pathogens within the gut microbiome, including *Salmonella*. Multiple studies have found that the composition of the poultry gut microbiome changes during *Salmonella* infection in human or in animals due to inflammation and an influx of phagocytes and lymphocytes from the circulation (Barman et al., 2008; Juricova et al., 2013; Stecher et al., 2007; Thiennimitr et al., 2012).

While a majority of poultry produced in the United States is done using conventional, industrial management techniques, alternative management systems are becoming more prevalent. For a variety of factors, including the attractiveness of being produced and marketed locally for retail, pastured raised chicken continues to be a popular market option for poultry goods (Gifford and Bernard, 2011; O'Bryan et al., 2019; Rainey et al., 2011). As the production of pastured poultry increases, food safety concerns must be taken into account. Pastured poultry operations are highly diverse in management practices (Jacob et al., 2008). Given this and combined with diverse living conditions, food safety problems can be somewhat unpredictable, presenting a challenge for limiting the prevalence of foodborne pathogens (Sirsat et al., 2009). The purpose of this study is to investigate the connection between pastured-related microbiomes and *Salmonella* presence on pastured poultry farms, as well as identifying bacterial taxa that have protagonistic or antagonistic associations with *Salmonella*.

7.2 Materials and methods

From March 2014 to November 2017, samples of feces, soil, and chicken carcass rinse were taken from 42 flocks on 11 pastured poultry farms in the southern United States. In the trial, all of the birds raised on pasture were broilers. The following kinds of samples were taken from each flock: (i) feces, (ii) pasture soil, (iii) ceca samples, (iv) whole carcass rinse immediately after processing (WCR-P), and (v) final product whole carcass rinse after chilling and storage time (WCR-F). There were a total of 1641 samples, comprised of 543 fecal samples, 177 ceca samples, 542 soil samples, 199 WCR-P samples, and 180 WCR-F samples.

7.2.1 Sample preparation

Three feces and soil samples were collected during the lifetime of a flock: (i) a few days after the flock was put on pasture (Start), (ii) midway through their stay on pasture (Middle), and (iii) on the day the flock was processed (End). On each day of sampling, the pasture was divided into five sections. Then, the five subsamples from each region were pooled to form a single sample (five soil samples and five feces samples were collected on each sampling day). Using sterile scoops, soil samples were collected from the top 0–7 cm of soil, and feces samples were collected from freshly deposited droppings on the soil surface. Gloves and scoops were switched between sample types and sampling locations. On the day of the on-farm slaughter, samples of the carcass rinse and ceca were obtained. Five eviscerated corpses had their cecal sacs removed and placed in a single sampling bag to create a pooled sample. There was a total of five pooled samples produced (n = 5). Gloves and scissors were changed between each combined sample. Each of the 25 corpses was put in a sterile plastic bag and then washed with 100ml of 10mM phosphate-buffered saline (PBS) and forcefully shaken for one minute. The WCR from five corpses were combined in a filtered stomacher bag to produce five pooled samples (n = 25).

7.2.2 *Salmonella* enumeration

The techniques of cultural isolation were taken from Rothrock et al. (2016). For pre-enrichment, the stomached homogenates were incubated overnight at 35 °C in filtered stomacher bags. *Salmonella* was isolated from environmental samples using two distinct enrichment broths: tetrathionate (TT; Becton-Dickinson, Sparks, MD) broth and Rappaport-Vassiliadis (RV; Becton Dickinson). 0.5 mL of pre-enrichment was placed into 9.5 mL of TT, and 0.1 mL was transferred into 9.9 mL of RV. One loopful (10 l) of each enrichment broth was streaked on two distinct differential media: brilliant green sulfa with novobiocin (BGS; Becton Dickinson) agar and xylose lysine tergitol-4 (XLT-4; Becton Dickinson). These plates were incubated at 35 °C overnight. Three *Salmonella*-like colonies per subsample were identified and verified using triple sugar iron agar (TSI; Becton-Dickinson) and lysine iron agar fermentation (LIA; Becton-Dickinson) after 18-24 h of incubation at 35 °C. *Salmonella* polyvalent O antiserum agglutination (Becton-Dickinson) was employed according to the manufacturer's instructions to confirm questionable TSI/LIA isolates.

7.2.3 DNA extraction and quantification

The DNA was extracted from 0.33 g of feces, 0.33 g of soil, 0.5 ml of cecal homogenate, and 0.5 ml of WCR. DNA was isolated from samples using a semi-automated hybrid DNA extraction procedure previously published (Rothrock et al., 2014). This approach was a mix of a mechanical technique using the FastDNA Spin Kit for Feces (MP Biomedicals, Solon, OH, USA) and an enzymatic technique based on the QIAamp DNA Stool Mini Kit (QIAGEN, Valencia, CA). Using the DNA Stool—Human Stool—Pathogen Detection Protocol of the QIAcube Robotic Workstation, DNA was purified. After purification, the DNA concentration in each sample was determined spectrophotometrically using the Take3 plate and the Synergy H4multimode plate reader (BioTek,Winooski, VT). For the microbiome technique, as indicated quantitatively in the second row of Table 2, samples were categorized as Salmonella-positive if the relative abundance of taxon *Salmonella* was more than zero, and *Salmonella*-negative otherwise.

7.2.4 16S rRNA gene sequencing

The Earth Microbiome Project Laboratory at the U.S. Department of Energy's Argonne National Laboratory constructed and sequenced the library (Argonne, IL, USA). The hypervariable V4 domain of the bacterial 16S rRNA gene was amplified using the F515 (5′-CACGGTCGKCGG CGCCATT-3′) and R806 (5′-GGACTACHVGGGTWTCT AAT-3′) primer set, with each primer containing Illumina adapter regions (Illumina, Inc., San Diego, CA, USA) and the reverse primer containing the Golay barcodes to facilitate multiplexing (Caporaso et al., 2011).Initial demultiplexing and quality filtering were performed using the QIIME pipeline version 1.9.1. (Caporaso et al., 2010b).

The 16S rRNA gene amplicon pool was demultiplexed using internal barcodes and primers were deleted in order to identify individual samples. The sequences were then compared to the Greengenes 13 8 database for chimeras and grouped into Operational Taxonomic Units (OTUs) based on 97% similarity criteria (DeSantis et al., 2006). Utilization of the open-reference OTU selection procedure. Using UCLUST and the Greengenes 13 8 database, a representative sequence was picked for each OTU and used for taxonomy classification. The sequences were aligned and filtered using PyNAST (Caporaso et al., 2010a). Following the construction of a phylogenetic tree, singleton OTUs and OTUs whose representative sequences could not be aligned using PyNAST were discarded.

7.2.5 Statistical analysis

The OTU counts for each sample were normalized so that the total summed over all taxa is one. In the following analyses, the relative abundance values were used. There were 1824 different taxa identified in total. To avoid the problem of sparsity with many zeros, the mean relative abundance of each OTU was calculated, and the mean OTU greater than the 75 percentile was retained. That is, the 456 most abundant OTUs were retained within each of the five sample types. 99.9% of the relative abundance remained after filtration. The analyses were carried out at the genus level.

Salmonella positive and negative samples were identified by cultural method (section 2.2) and microbiome method (section 2.3). Linear discriminant analysis effect size (LEfSe) (http://huttenhower.sph.harvard.edu/galaxy/) was used to identify genomic features that distinguish between groups (Segata et al., 2011). To visualize the different taxa, the linear discriminant analysis (LDA) score was calculated, and a taxonomic cladogram was constructed. An LDA score greater than four was used as threshold for the LEfSe analysis. The LEfSe algorithm uses Kruskal-Wallis test ($\alpha = 0.05$) to check difference between samples (*Salmonella* positive and negative samples). To compare cultural method and microbiome method, sensitivity, specificity, and accuracy were calculated. In this calculation, cultural method was set as reference method:

$$Sensitivity = \frac{TP}{TP+FN} \quad (1)$$

$$Specificity = \frac{TN}{TN+FP} \quad (2)$$

$$Accuracy = \frac{TP+TN}{TP+FN+TN+FP} \quad (3)$$

where TP is true positive, FN is false negative, TN is true negative, and FP is false positive. Sensitivity is the ability of a method to correctly classify true positive, where specificity is the ability of a method to correctly classify true negative (Parikh et al., 2008). Sensitivity also measures the rate of a method to incorrectly classify negative samples as positive samples (false negative), while specificity is the rate at which a method incorrectly classifies positive samples as negative samples (false positive). In our circumstances, a higher specificity value is more important than a higher sensitivity value because failing to detect positive samples increase the risks of food safety.

7.3 Results and Discussion

Salmonella prevalence by sample type is shown in Table 7.1. In fecal samples, *Salmonella* prevalence was 17.9% and 39.9% as assessed by cultural and microbiome methods, respectively. Soil samples showed the largest difference between cultural (13.7%) and microbiome (54.4%) methods in *Salmonella* prevalence. In WCR-P and WCR-F samples, *Salmonella* prevalence were lower in cultural method (26.1% and 21.1%, respectively) compared to microbiome method (43.2% and 50.0%, respectively). In general, we noticed that the cultural method showed lower proportion of positive samples across the sample types compared to microbiome method. This may be due to some of *Salmonella* cells entered viable but non-culturable state (VBNC). Pathogenic bacteria can go into a survival state in which they no longer capable to form colonies on media when exposed to unfavorable conditions (Pinto et al., 2015). They are intact cells that have changed in morphology (Takeda, 2011) and cell wall composition (Signoretto et al., 2002) but can still perform respiration (Oliver and Bockian, 1995), gene transcription (Lleo et al., 2000), and other functions. To identify VBNC cells of *Salmonella* spp., RNA-based genotypic approaches targeting stress-related genes were used (Ramamurthy et al., 2014). This may explain higher *Salmonella* prevalence were observed using microbiome method than cultural method in all sample types.

Table 7.2 summarizes the sensitivity, specificity, and accuracy of five sample types using the cultural method as the reference and the microbiome method as the testing method. In our results, the sensitivities of pre-harvest samples were 0.423 and 0.676 (feces and soil, respectively), which indicates that the microbiome method classified 42.3% and 67.6% *Salmonella* feces and soil samples that were also determined to be *Salmonella* positive culturally. These results indicate that the number of false negative samples was high for feces samples and low for soil samples, suggesting that microbiome method favors correctly identifying *Salmonella* positive samples for soil samples, but may not for feces samples. Grim et al. (2017) reported 100% sensitivity in detecting *Salmonella* in cilantro, ice cream, and chili powder when 0.01% relative abundance threshold. However, the specificities of soil samples were 0.476, indicating that there is a high chance of microbiome method to incorrectly identify *Salmonella* positive samples as negative samples. Grim et al. (2017) also reported low specificity in cilantro and chili powder samples. The author found high abundance of *Enterobacter* species in false positive samples. This high diversity of closely related species may have resulted in the formation of low frequency chimeras during PCR that were not easily identified during preprocessing and may have been assigned to *Salmonella* (Grim et al., 2017). For ceca samples, the sensitivity was 0.135 though the specificity is 0.660, which means microbiome method correctly identified 13.5% of *Salmonella* positive samples and 66.0% of *Salmonella* negative samples. For WCR-P and WCR-F samples, the specificities were 0.551 and 0.500, respectively, indicating that the microbiome method correctly identified 55.1% and 50.0% of true negative samples. The relatively low specificity indicates that there is a high likelihood of misclassifying *Salmonella* positive samples as negative samples. In terms of accuracy, higher accuracy means better performance of the testing method (microbiome method) compared to the reference method (cultural method). The highest accuracy was achieved in ceca samples and the lowest accuracy was in WCR-F samples. In summary, microbiome method showed relative low accuracy in terms of cultural methods. Furthermore, the likelihood of incorrectly classifying *Salmonella* negative samples as positive samples was high by microbiome method.

3.1 LEfSe analysis for pre-harvest samples

Firmicutes and *Proteobacteria* accounted for 85% of the taxa in fecal microbiomes, whereas these 2 phyla only accounted for 55% of the taxa in soil samples (Fig. 7.1). For WCR-P and WCR-F samples, they shared similar microbial composition patterns. Table 3 shows the top five most frequent geuns-level taxa across all sample types. The top five OTUs accounted for 72% of the relative abundance in feces samples. For soil samples, the top five taxa accounted for less than 20% of the relative abundance, while the top five OTUs accounted for 44% to 49% of the relative abundance in the three post-harvest samples. *Lactobacillus* was the most common taxon in both pre-harvest samples (feces and soil), with relative abundance values of 0.526 and 0.065, respectively. Different from other sample types, the top three most common taxa were

Ruminococcaceae (0.148), *Clostridiales* (0.119), and *Streptophyta* (0.069) in cecal samples. *Acinetobacter* were the most common microbial taxa in WCR-P and WCR-F samples (0.173 and 0.165, respectively) and represented the only taxon in the five most abundant taxa in every sample type.

In this study, we wanted to know what other taxa in the microbiome data correlated with *Salmonella* positive and negative samples, in addition to comparing the overall efficacy of using cultural versus microbiome-based quantification of *Salmonella*. The operational taxonomic units (OTUs) that are most likely to explain the differences between classes are found using the linear discriminant analysis (LDA) effect size (LEfSe) method, which combines additional tests encoding biological consistency and effect relevance with standard tests for statistical significance (Segata et al., 2011). The advantage of LEfSe is that it can reliably calculate statistically significant differences (expressed as LDA scores) across various situations while also recognizing the hierarchy of the taxonomic classification (Munoz-Benavent et al., 2020).

7.3.1.1 Feces samples

LEfSe results are shown for cultural (Fig. 7.2A) and microbiome methods (Fig. 7.2B) of feces samples. *Proteobacteria* and *Gamma-proteobacteria* were the top two taxa that were significantly enriched in *Salmonella* positive samples when assessing it molecularly. This result agrees with Oh et al. (2017a) and Li et al. (2017) that *S*. Gllinarum infection resulted in an increase of *Proteobacteria* and a decrease in *Firmicutes*. Similarly, the abundance of *Proteobacteria* increased in *Salmonella enterica* serovar Typhimurium infected broilers (Zhang et al., 2020). These microbial changes encourage the growth of enteropathogenic bacteria while inhibiting the growth of anaerobic and short-chain fatty acid-producing bacteria (Choi et al., 2015). *Pseudomonadales* was consistently enriched in *Salmonella* positive samples using the microbiome method. Similarly, Oh et al. (2017a) reported an increase in *Pseudomonas* abundance after *Salmonella* infection. *Enterobacteriaceae* was selected by microbiome method to represent *Salmonella* positive samples. Similarly, *Enterobacteriaceae* was found to be increased from 2.9% to 10.9% in *Salmonella* infected samples (Oh et al., 2017b).

Taxa related to *Lactobacillaceae* (order), *Lactobacillales* (family), *Lactobacillus* (genus), and *Firmicutes* differentiated *Salmonella* negative samples from positive samples by cultural and microbiome methods. Previous work has shown that the abundance of *Lactobacillus* ranged from 42.7% to 49.0% from chickens before *Salmonella* infection ,which is in line with our results (Oh et al., 2017a). Several strains of *Lactobacillus* have been reported to be beneficial to their hosts (Sun et al., 2015). The beneficial effects of probiotic strains include the production of antimicrobials such as hydrogen peroxide, lactic acid, and bacteriocin (Makarova et al., 2006). The benefits also include the production of enzymes for the breakdown of feed-based polysaccharides, and immunomodulation (Lee et al., 2013; Pacheco and Sperandio, 2015; Pajarillo et al., 2015; van den Nieuwboer et al., 2016). *Lactobacilli* at high levels have been shown to inhibit the growth of *Salmonella, Escherichia coli* K88, and several other pathogens in broiler chickens, thereby improving livestock performance by protecting against bacterial infection (Oh et al., 2017b).

Planococcaceae and *Bacillales* were the common taxa that were significantly abundant in *Salmonella* positive samples by the two detection methods. Previous *Salmonella*-based work has shown that *Planococcaceae* was more abundant in non-infected samples than in *Salmonella* Enteritidis infected samples at 3- and 7-days post-infection (Mon et al., 2020). Zenner et al. (2021) reported higher *Bacillales* abundance in non-pathogen infected groups than in infected groups. This may because *Bacillales* protect the host gut from bacterial invasion and intestinal pathologies (Ilinskaya et al., 2017). However, in our results both *Planococcaceae* and *Bacillales* was associated with the presence of *Salmonella*, but since both detection methods identified these associations, more work needs to be done to determine the underlying cause of this protagonistic relationship to *Salmonella*.

7.3.1.2 Soil samples

The soil microbiome is a diverse ecological system that plays a direct role in the maintenance of important soil functions. On pastured poultry farms, rotationally grazed system (the system consists of strategically rotating livestock through paddocks to maximize forage productivity) is applied. Pasture management practices influence the soil nutrient status and consequently may affect soil bacterial communities (Yang et al., 2019). Figure 7.3A and 7.3B display taxa selected to differentiate *Salmonella* positive and negative samples in soil by cultural and microbiome methods, respectively. *Gamma-proteobacteria* was the common feature selected by both detection methods in *Salmonella* positive samples, where *Proteobacteria* was the top taxon enriched in *Salmonella* positive samples according to the microbiome method. Several studies have reported *Proteobacteria* as the dominant phylum in chicken housing soil or pastured soil, suggesting their central role in the soil ecosystem (Crippen et al., 2019; Parente et al., 2021; Yang et al., 2019). *Bacteroidetes* was another dominant phylum (makes up an average of 5% of soil microbiome) in soil samples (Janssen, 2006), which was also significantly enriched in *Salmonella* positive samples. This phylum was also largely associated with the internal and external flora of chickens, indicating that some members of *Bacteroidetes* were passed from feces from chickens (Shange et al., 2013). *Acidobacteria* was found to be significantly abundant in *Salmonella* negative samples. *Acidobacteria* may decrease under greater precipitation and under the presence of *Proteobacteria* (Castro et al., 2010). According to Sait et al. (2006), the pH of soil samples strongly influenced the cultivation of *Acidobacteria* colonies, with growth favored by mild to moderate acidic conditions.

7.3.2 LEfSe analysis for post-harvest samples

7.3.2.1 Ceca samples

The LEfSe results by cultural (Fig. 7.4A) and microbiome method (Fig. 7.4B) in ceca samples showed that only one taxa related to *Actinobacteria* was significantly enriched in *Salmonella* positive samples by cultural method. Mon et al. (2015) and Hernandez-Patlan et al. (2019) reported higher *Actinobacteria* abundance in *S*. Enteritidis infected samples than in control samples. Similarly, *S. ser.* Enteritidis infection resulted in the increased abundance of *Actinobacteria* in ceca (Li et al., 2021).

Proteobacteria and *Epsilon-proteobacteria* were significantly enriched in *Salmonella* positive samples according to the microbiome method. Indeed, *Proteobacteria* have been linked to *S.* enteritidis infection in animals (Sekirov et al., 2010; Stecher et al., 2007). Chang et al. (2020) found higher *Proteobacteria* abundance in *Salmonella enterica* subsp. *enterica* infected chickens. The increased abundance of *Proteobacteria* in *Salmonella*-infected broilers suggests dysbiosis and imbalance in the gastrointestinal tract. Stecher et al. (2007) reported that *Proteobacteria* may benefit from inflammation caused by *Salmonella* infection; however, Liu et al. (2018) reported a decrease of *Proteobacteria* and an increase of *Firmicutes* after *S.* Typhimurium infection.

Campylobacter and its family and order were significantly associated with *Salmonella* positive samples, suggesting a possible correlation between the two major poultry-related bacterial foodborne pathogens. Jacobs-Reitsma et al. (1994) stated that *Campylobacter* was positively correlated with *Salmonella* within broiler flocks; however, Hue et al. (2011)

investigated *Campylobacter* and *Salmonella* levels in ceca and reported no correlation between these two pathogens. Given the stringent growth conditions of *Campylobacter* in culture, the microbiome-related detection method may be allowing us to observe previously unaccounted for associations.

7.3.2.2 WCR samples

Figure 7.5 shows that taxa related to *Pseudomonadales* and *Gamma-proteobacteria* were significantly enriched in *Salmonella* positive WCR-P samples by both cultural and microbiome detection methods. Pourabedin et al. (2017) reported *Pseudomonadales* was overrepresented in *S*. Enterititids infected chickens. Similarly, Zhang et al. (2021a) found that *Pseudomonadales* was significantly increased with *S*. Typhimurium challenged chickens. Previously, *Pseudomonadales* has been related to harmful gut microorganisms that induce a variety of disorders of the host (Wang et al., 2017). It has been detected in conveyor belt in chicken processing plants (Song et al., 2021). Aside from *Pseudomonas*, *Proteobacteria* was also a dominant phylum in chicken meat (Cesare et al., 2018b; Rouger et al., 2018; Song et al., 2021).

Enterobacterales and *Enterobacteriaceae* were identified as the third and fourth most enriched taxa in WCR-P samples by microbiome method (Fig. 7.5B). The *Enterobacteriaceae* family contains pathogens such as *E. coli* O157 and *Salmonella* spp. (Whyte et al., 2004). Handley et al. (2018) found *Enterobacteriaceae* to be consistently abundant in chicken carcasses during processing, suggesting it could be a potential indicator microorganism for quality and cleanness. *Moraxellaceae* and *Firmicutes* were the second and third taxa selected to represent *Salmonella* positive samples using the cultural method. One study reported high abundance of *Firmicutes* and *Moraxellaceae* on the skin of chicken carcasses (Cesare et al., 2018a). *Moraxellaceae* family includes mild spoilage bacteria such as *Acinetobacter* and *Psychrobacter* for dairy and meat product due to their lipolytic activity and lack of proteolytic activity (Dourou et al., 2021).

Taxa related to *Cyanobacteria*, Chloroplast, and *Streptophyta* were the common features that significantly represented *Salmonella* negative samples in WCR-P samples by both detection methods. Zhang et al. (2021b) investigated chilled chicken samples during processing and found *Cyanobacteria* was one of the major phyla. However, He et al. (2020) found that *S*. Typhimurium infection induced the increase of *Cyanobacteria* in abundance, which can synthesize toxins to produce adverse effects on animals. Johnson et al. (2018) the presence of *Cyanobacteria* may also represent Chloroplast DNA from plant material in feed, which in turn explained the high abundance of Chloroplast in WCR-P samples.

Figure 7.6B shows that *Proteobacteria* was the number one taxon that was significantly enriched in *Salmonella* positive WCR-F samples, as assessed by the microbiome method. *Proteobacteria* is the phylum included *Enterobacterales* (order) and *Enterobacteriaceae* (family), which is a large family of Gram-negative bacteria that contains pathogens such as *Salmonella*, *E. coli, Shigella,* and *Yersinia* (Kim et al., 2018). Taxa related to *Enterobacterales* and *Enterobacteriaceae* were enriched in *Salmonella* positive WCR-F samples according to tge microbiome method. *Enterobacteriaceae* was one of the main phyla of chicken meat during packaging (Marmion et al., 2021).

7.4 Conclusions

In conclusion, this study demonstrated that the cultural detection method showed lower *Salmonella* prevalence than did the microbiome method in all sample types, and there were differences in agreement between the two detection methods mased on sample type. Additionally, taxa that significantly differentiated *Salmonella* positive and negative samples based on cultural and microbiome detection methods were identified. In general, the LEfSe comparison of two methods showed better agreement for pre-harvest samples than for post-harvest samples. *Planococcaceae* and *Bacillales* were identified as the common taxa selected to represent *Salmonella* positive fecal samples, while *Firmicutes* and *Lactobacillus* were the common taxa significantly abundant in *Salmonella* negative fecal samples. *Pseudomonadales* and *Gamma-proteobacteria* were the common taxa significantly enriched in *Salmonella* positive samples in WCR-P samples. *Streptophyta*, *Cyanobacteria*, and Chloroplasts were significantly enriched in *Salmonella* negative WCR-P samples. This study provided the comparison of cultural and microbiome method to identifying *Salmonella* presence in pastured poultry farms. Taxa significantly associated with *Salmonella* positive or negative samples were detected, providing data for future research of *Salmonella* correlated taxa which could potentially be used to biologically exclude *Salmonella* at different stages of the poultry farm-to-fork continuum.

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Table 7.1 *Salmonella* prevalence for cultural and microbiome methods by sample types

	Feces	Soil	Ceca	WCR-P	WCR-F
Cultural method	17.9%	13.7%	13.6%	26.1%	21.1%
Microbiome method	39.9%	54.4%	31.1%	43.2%	50.0%
Sample size	542	542	177	199	180

Table 7.2 Sensitivity, specificity, and accuracy based on sample types

	Feces	Soil	Ceca	WCR-P	WCR-F
Sensitivity	0.423	0.676	0.135	0.385	0.500
Specificity	0.605	0.476	0.660	0.551	0.500
Accuracy	0.573	0.504	0.588	0.506	0.500

Table 7.3 The top five most frequent taxa (relative abundance) by sample types

Taxa	Feces	Soil	Ceca	WCR-P	WCR-F
#1	*Lactobacillus* (0.526)	*Lactobacillus* (0.065)	*Ruminococcaceae* (0.148)	*Acinetobacter* (0.173)	*Acinetobacter* (0.165)
#2	*Acinetobacter* (0.070)	*Acinetobacter* (0.048)	*Clostridiales* (0.119)	*Streptophyta* (0.168)	*Streptophyta* (0.134)
#3	*Enterobacteriaceae* (0.048)	*Bacillus* (0.034)	*Streptophyta* (0.069)	*Lactobacillus* (0.067)	*Lactobacillus* (0.059)
#4	*Streptococcus* (0.041)	*Chitinophagaceae* (0.027)	*Acinetobacter* (0.053)	*Lactococcus* (0.041)	*Enterobacteriaceae* (0.058)
#5	*Enterococcus* (0.033)	*Enterobacteriaceae* (0.023)	*Oscillospira* (0.048)	*Enterobacteriaceae* (0.039)	*Lactococcus* (0.053)

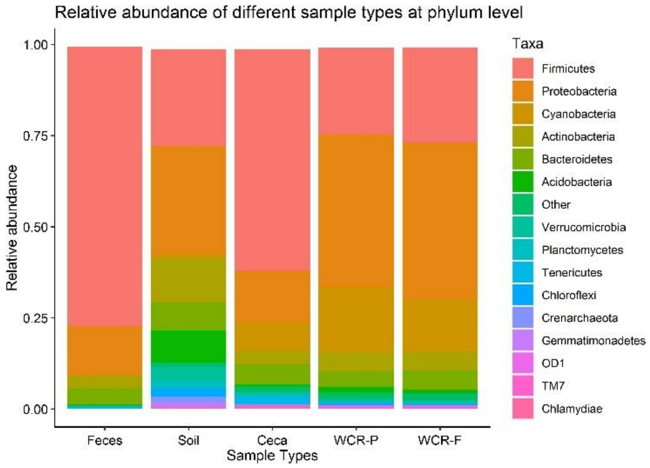

Figure 7.1 Bar plot of relative abundance by sample types at phylum level

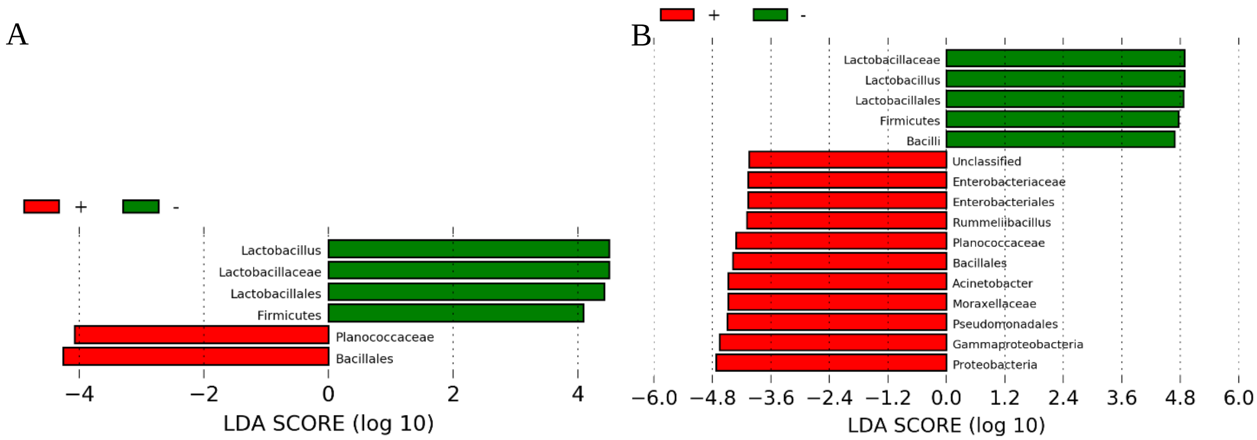

Figure 7.2 Fecal LEfSe comparison between *Salmonella* positive (red) and negative (green) samples as assessed by the cultural method (A) or the microbiome method (B)

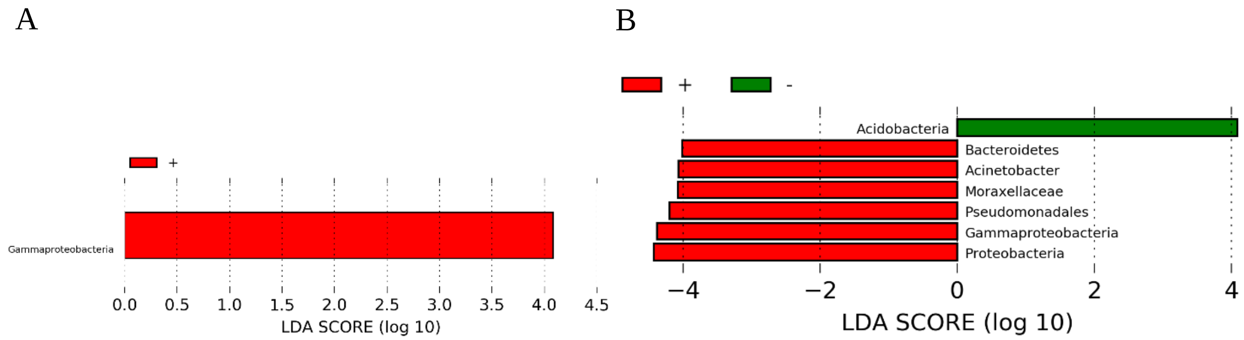

Figure 7.3 Soil LEfSe comparison between *Salmonella* positive (red) and negative (green) samples as assessed by the cultural method (A) or the microbiome method (B)

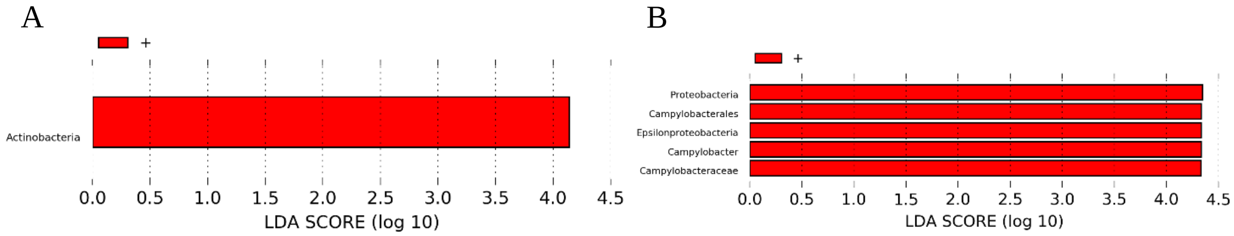

Figure 7.4 Cecal LEfSe comparison between *Salmonella* positive (red) and negative (green) samples as assessed by the cultural method (A) or the microbiome method (B)

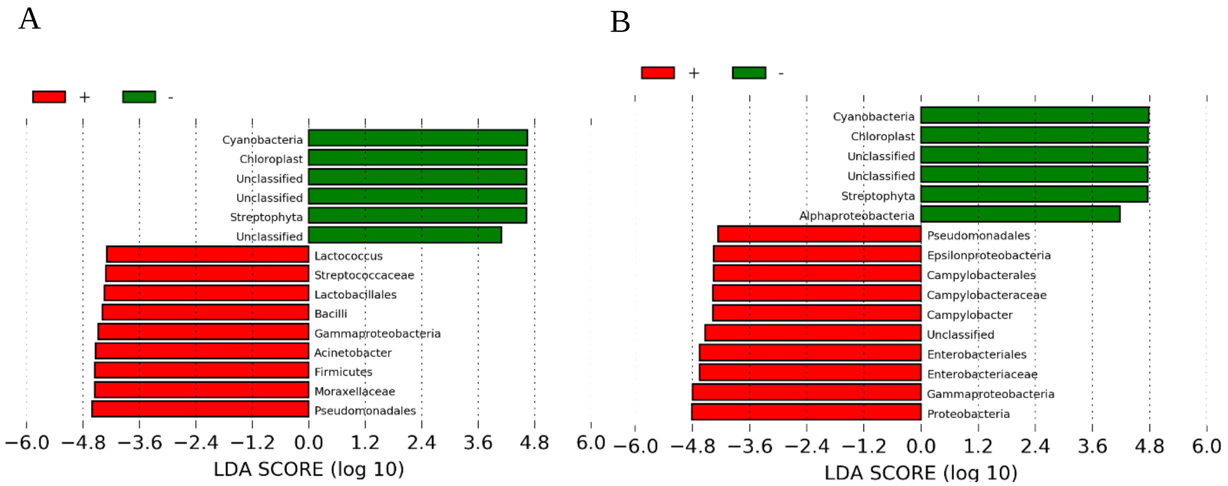

Figure 7.5 Whole carcass rinse after processing (WCR-P) LEfSe comparison between *Salmonella* positive (red) and negative (green) samples as assessed by the cultural method (A) or the microbiome method (B)

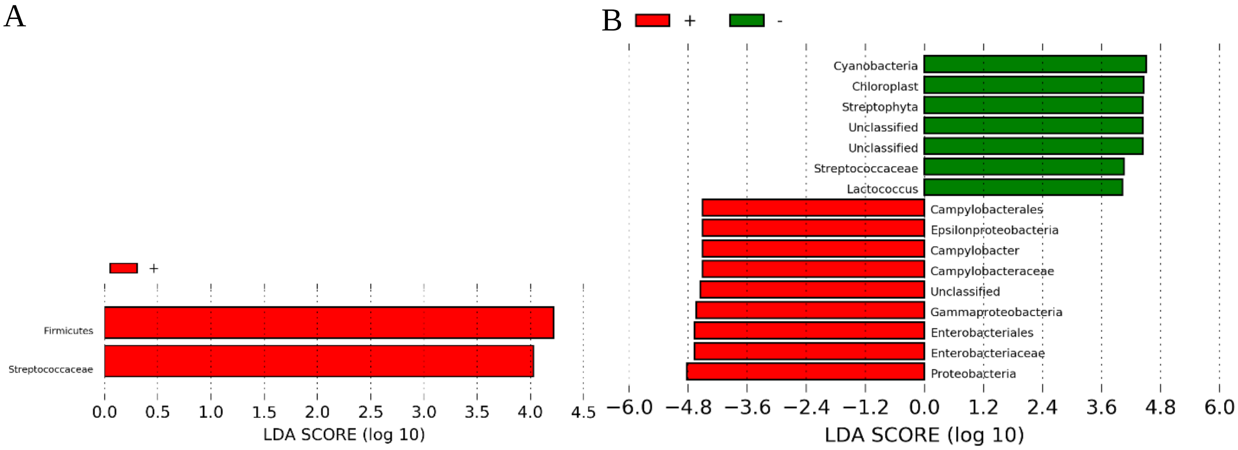

Figure 7.6 Final product whole carcass rinse (WCR-F) LEfSe comparison between *Salmonella* positive (red) and negative (green) samples as assessed by the cultural method (A) or the microbiome method (B)

CHAPTER 8

ASSESSING THE RISK OF SEASONAL EFFECTS OF *CAMPYLOBACTER* CONTAMINATED BROILER MEAT PREPARED IN-HOME IN THE UNITED STATES

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[1] Xu, X., Rothrock Jr, Kumar, G. D., & Mishra, A. (2022). Submitted to *Microbial Risk Assessment*, 10/18/2022

Abstract

Campylobacter has long presented a major problem for the food safety of broiler meat. The goal of this study was to develop a retail-to-consumption quantitative microbial risk assessment model that could be used to estimate the seasonal risk of campylobacteriosis from consuming broiler meat in the United States. Data were taken and used to make distributions that could be used to estimate *Campylobacter* growth during retail storage, transportation, and home storage, as well as changes in concentration during preparation and due to cross-contamination. A Monte Carlo simulation with 100,000 iterations was used to estimate the risk of infection per serving and the number of infections in the United States by season. Sensitivity analyses were done to find out which factors were strongly linked to a higher risk of campylobacteriosis. In the summer, chicken meat was estimated to have a median risk of infection per serving of 9.22×10^{-7} and cause an average of about 27,058,680 infections. During the winter months, the median risk of infection per serving was estimated to be 4.06×10^{-7} and cause an average of about 12,085,638 infections. The sensitivity analysis concluded that not washing hands before preparation is the most important risk factor. The "what-if" scenario analysis found that thawing chicken at room temperature greatly increased the chance of getting *Campylobacter* infections and getting sick. The risk assessment model provides information about the risk of broiler meat to public health by season. These results will help understand the most important steps to reduce the food safety risks from contaminated chicken products.

8.1. Introduction

In the chicken sector, *Campylobacter* spp. presents a persistent food safety risk. According to the Centers for Disease Control and Prevention (CDC), there were 96 campylobacteriosis outbreaks linked to the eating of chicken in the United States between 1998 and 2020, resulting in 856 illnesses (CDC, 2020). *Campylobacter* has been demonstrated to reside in the gastrointestinal tract of broilers, which may explain why the bacterium is so commonly connected with poultry-related campylobacteriosis (Hermans et al., 2012). *Campylobacter* may infect broilers and chicken carcasses at any stage of the broiler supply chain. Examples of pre-harvest contamination include feed, other farm animals, biosecurity hazards (wildlife species), potable water, soil, insects, farm equipment, personnel, visitors, and farm vehicles (Ghareeb et al., 2019). Postharvest contamination is caused by fecal contamination of feathers and skin during transit, fecal material leaks during evisceration, and contact with contaminated equipment and water (Keener et al., 2004).

Multiple studies have shown substantial seasonal trends in the incidence of *Campylobacter* in the environment and at different levels of the food chain between live animals and human sickness. Stern (1995) showed that *Campylobacter* concentrations in the United States were lower in the autumn and spring than in the summer and winter. Willis and Murray (1997) observed that from May to October in the United States, the incidence of *Campylobacter* is high (86.7% to 96.7%) based on a one-year study of carcass samples. In addition, research from France, the United Kingdom, and several other nations indicate a greater *Campylobacter* frequency in warm months than in cold ones (Allain et al., 2014; Jorgensen et al., 2011; Nylen et al., 2002). Several studies have also linked human campylobacteriosis incidences to the hottest months of the year (Louis et al., 2005; Miller et al., 2004).

Since the late 1990s, when Willis and Murray (1997) utilized quantitative microbial risk assessment (QMRA) to estimate the risk of salmonellosis due to consumption of liquid eggs, QMRA has been a commonly used method in the food industry to evaluate the risk of microbiological hazards to food consumers. Quantitative microbial risk assessment (QMRA) is a technique for estimating human health risks based on dose-response (DR) models for particular (reference) pathogens and exposure scenario evaluations (Haas et al., 2014). The process consists primarily of determining the concentration of reference pathogens at the points of environmental exposure, typically by estimating the sources and modeling pathogen fate and transport to the points of human exposure; this concentration is then combined with ingestion volume to calculate the dose. Dogan et al. (2019) quantified the risk of *Campylobacter* during processing in the United States; Lindqvist and Lindblad (2008) summarized the risk of *Campylobacter* during the handling of raw chicken in Sweden; and Hartnett et al. (2001) developed a model to assess the risk of *Campylobacter* at the point of slaughter in the United Kingdom. The purpose of this research was to develop a retail-to-consumption QMRA model that could be used to evaluate the seasonal impact of the yearly illnesses induced by the eating of broiler meat processed at home in the United States.

8.2. Materials and methods

8.2.1 QMRA overview

A process flow from the moment that broiler meat is packed till its consumption by customers has been devised (Figure 8.1). The flow illustrates the human consumption of a portion of meat cooked from a retail-purchased package of chicken. The flow includes retail storage, delivery to the consumer's house, and storage, preparation, and consumption at the consumer's residence. A search of the scientific literature was conducted to discover distributions that might be used to explain characteristics in each of these domains, as well as the growth and inactivation kinetics of *Campylobacter* at different temperatures. Table 8.1 presents the variables used in the QMRA model.

8.2.2 *Campylobacter* growth kinetics

To capture the growth behavior of *Campylobacter* at the vast range of temperatures it may experience along the retail-to-consumption chain, a thorough knowledge of *Campylobacter* growth rates on broiler meat is required. Primary growth data were identified from Blankenship (1982), Nicorescu and Crivineanu (2009); Solow et al. (2003). Only a limited number of studies were found investigating the growth of *Campylobacter* in chicken meat under various temperatures. For each research, primary growth data were extracted, and the three-phase linear model was fitted to the growth data in order to calculate the specific growth rate (k) using the following equations (Buchanan et al., 1997)

$$y_t = y_0 \text{ for } t \le t_{lag} \quad (1)$$

$$y_t = y_0 + k(t - t_{lag}) \text{ for } t_{lag} < t < t_{max} \quad (2)$$

$$y_t = y_{max} \text{ for } t \ge t_{max} \quad (3)$$

Where y_t is the population of bacteria at time t (log CFU/g); y_0 is the initial population of bacteria (log CFU/g); y_{max} is the maximum population of bacteria supported by the environment (log CFU/g); k is the specific growth rate (log CFU/h); t is the elapsed time (h); t_{lag} is the lag time (h); t_{max} is the time when y_{max} is reached (h). When there were only two phases in the growth data, a biphasic model was fitted, with the phases consisting of either lag phase and exponential phase or exponential phase and stationary phase. Primary models were fitted using the United States Department of Agriculture (USDA) Integrated Pathogen Modeling Program (IPMP; Version 2013) (Huang, 2014).

After estimating k from primary data, the Ratkowsky model was applied to the growth rates as described by the following equation (Ratkowsky et al., 1982):

$$\sqrt{k} = b(T - T_{\min}) \quad (4)$$

Where T is the temperature (°C); $T_{\min}$ is the theoretical minimum temperature for growth (°C); and b is a growth constant. It has been shown that the Ratkowsky model should be used to temperatures between the lowest and optimal growth temperatures of an organism; hence, only growth rates from temperatures between 37 and 42 °C were employed. Hazeleger et al. (1998) and Park (2002) showed that *Campylobacter* required a minimum growth temperature of 31°C. Therefore, if simulated temperatures in the QMRA were below 31°C, a growth rate of zero was used to indicate no growth. MATLAB Curve Fitting Toolbox (Version R2019b; Mathworks, Natick, MA) was used to conduct secondary modeling, and estimates for b and $T_{\min}$ were derived. Because of lacking growth data for *Campylobacter* on chicken meat, single estimated values were used in QMRA model.

8.2.3 Product temperature change

Newton's law of heating has been used to explain the change in temperature of a food product when it enters a warmer ambient environment as a function of the product's initial temperature, the ambient temperature, and the amount of time the product spends in the ambient temperature (Mishra et al., 2016). It can be described by the following equation:

$$T = T_a - (T_a - T_0)e^{-Bt} \tag{5}$$

Where T is final product temperature (°C); T_a is ambient temperature (°C); T_0 is starting product temperature (°C); t is time in ambient temperature (h); and B is a constant (h^{-1}). Using equation 5, equation 4 is re-written to describe the growth rate of *Campylobacter* when chicken enters a warmer ambient temperature (equation 6).

$$\sqrt{\mu} = b(T_a - (T_a - T_0)e^{-Bt} - T_{\min}) \tag{6}$$

The distribution of B is obtained from Golden and Mishra (2021). Equation 6 was used to predict the growth rate of *Campylobacter* when a shift from cold to warm ambient temperature was anticipated; otherwise, Equation 4 was utilized. Due to @Risk software restrictions, only one growth rate was generated for each iteration of the QMRA model, given the time and temperature experienced at that iteration.

8.2.4 Retail prevalence

Multiple studies reported monthly prevalence data for *Campylobacter* in chicken meat (Blankenship, 1982; Nicorescu and Crivineanu, 2009; Solow et al., 2003). Spring (March, April, and May), summer (June, July, and August), autumn (September, October, and November), and winter (December, January, and February) meteorological seasons are used in this study (Trenberth, 1983). By season, monthly prevalence statistics were categorized. Then, 1,000 samples were generated using the bootstrapping method. The mean values of each sample collected using bootstrapping approach was computed. The @RISK software was used to fit distributions to these mean values. *Campylobacter* initial concentration was determined based on a 2012 countrywide USDA study in which chicken parts were examined at the end of the manufacturing line and positive samples were measured in CFU/mL (USDA, 2012). Due to the availability of data, it is vital to mention that the concentration was based on chicken samples gathered after manufacturing and before reaching retail. Therefore, it was hypothesized that neither an increase nor a decrease in *Campylobacter* counts happened during transit from the manufacturing site to the retail location.

8.2.5 Retail storage

Growth of *Campylobacter* in chicken products were assessed by two parts: retail cold room storage time and temperatures and display storage time and temperatures (Derens et al., 2006). An exponential distribution was fit for cold room storage and display storage time. A normal distribution was fit for cold room storage and display temperature.

8.2.6 Transportation and home storage

The monthly average ambient daytime temperatures in the United States' major cities was obtained from CR (2022). In order to determine the average ambient daytime temperature per season, distributions were developed. Ecosure (2008) gathered data on transportation times and fitted them to a log-logistic distribution. The distribution was trimmed at the lowest and highest observed times to prevent impractical low and high values on either ends. The bacterial growth rate during transportation was approximated using equation 6, where the ambient temperature and time spent at the ambient temperature were selected from the distribution mentioned above and the initial temperature was determined by the retail storage temperature. In addition, a Beta general distribution was utilized to estimate the period between customers' arrival at home and the placement of meat products in the refrigerator (Mazengia et al., 2015). Using the ambient room temperature from Booten et al. (2017), the growth of *Campylobacter* was approximated prior to refrigeration.

In the baseline model, scenarios were built based on whether or not a customer elected to freeze the chicken meat they bought. In a study conducted by Mazengia et al. (2015), it was discovered that forty percent of respondents froze chicken meat before consuming it. In each model iteration, a Bernoulli distribution with $p = 0.40$ was utilized to determine whether or not a customer froze their chicken meat. If a customer did not freeze their meat, it was presumed that it was cooked immediately after being stored in the refrigerator. According to the same poll, consumers kept chicken meat in the refrigerator for an average of one to seven days before consumption (Mazengia et al., 2015). The time meat was held in the refrigerator prior to cooking or freezing was modeled using a Pareto distribution. If a customer chose to freeze their chicken, the same quantity of refrigerated storage was utilized to replicate the time it would take them to store the chicken in frozen storage. It was anticipated that no growth occurred during frozen storage since realistic freezer temperatures would not support *Campylobacter* to proliferate (Ritz et al., 2007). After frozen storage, four thawing methods were considered: refrigeration, running water, microwave, and room temperature. Extracted data from a survey performed by Mazengia et al. (2015) were used to assess the chance that a customer would utilize each method to defrost frozen food. The USDA recommends all methods other than room-temperature thawing for the safe defrosting of meat (USDA, 2013). Thawing timings for refrigeration and running water were based on USDA standards, while microwave thawing periods were based on common "defrost" settings (25-30% power) for residential microwaves (Taher and Farid, 2001; USDA, 2013). Growth rates were calculated based on thawing duration and temperatures experienced throughout the different thawing methods. For all preparation techniques, it was expected that customers would immediately cook their chicken after thawing.

8.2.7 Cross-contamination during preparation

In the baseline QMRA model, the following cross-contamination scenarios were evaluated: raw chicken to hands, raw chicken to utensils (e.g., cutting boards, knives, etc.), hands to cooked chicken, and infected utensils to cooked chicken. A number of studies have provided transfer rate data about *Enterobacter aerogenes* and *Campylobacter* spp. during food preparation (Chen et al., 2001; Luber et al., 2006; Verhoeff-Bakkenes et al., 2008). Although data on the transfer rates from raw chicken to hands and raw chicken to utensils were provided, the transfer rates from unclean hands and utensils to cooked chicken were calculated using lettuce, bread, and cucumber, since these information for chicken are not available. For each study, transfer rates were extracted, and distributions were fitted. We calculated the changes in *Campylobacter* concentration after each phase of handling. Kosa et al. (2015) estimated that 88.3 percent of individuals wash their hands after handling raw chicken. Therefore, a Bernoulli distribution was utilized to determine whether hand washing decrease should be applied to the hand concentration. Chen et al. (2001) provided statistics on the decrease of hand washing. If a person used different tools than those used to handle raw chicken, the transfer rate from utensils to cooked chicken was assumed to be 0%.

8.2.8 Cooking

The chicken meat cooking time and temperature data were collected from Oscar (2004) and Bruhn (2014), respectively. A Pert distribution was used to simulate the process of cooking chicken at home. In this stage, the baseline model examined whether or not the chicken product was undercooked. Undercooking is described as cooking chicken meat below the USDA-recommended temperature of 165 °F (73.9 °C) (USDA, 2013). According to a study conducted by Bruhn (2014) and Ecosure (2008), 39.9% of chicken products were undercooked. If chicken was adequately cooked, it was considered that the prepared product had 0 CFU/g *Campylobacter*. If chicken was undercooked, the D-value was calculated based on the inactivation model provided by van Asselt and Zwietering (2006). As higher temperatures were predicted to result in shorter cooking periods, a correlation coefficient of -0.75 was used to represent the link between cooking time and temperature (Golden and Mishra, 2021).

8.2.9 Dose-response modeling and risk characterization

Multiplying the concentration of *Campylobacter* in an eaten serving by the serving size yielded the ingested dosage. For all simulations, a serving size of 85 g was adopted based on the reference quantity commonly eaten per eating occasion for chicken meat (9 CFR 381.412) (CLS, 2020). As a final result, we intend to estimate the probability of infection and illness resulting from the consumption of chicken meat infected with *Campylobacter*. Consequently, the evaluation of exposure is dependent on the dose-response relationship. The most common dose-response relationship for *Campylobacter* is the beta Poisson model for infection probability (Medema et al., 1996; Teunis and Havelaar, 2000). The probability of infection was determined by the following equation:

$$P_{inf} = 1 - \left(1 + \frac{D}{\beta}\right)^{-\alpha} \tag{7}$$

Where P_{inf} is the probability of infection; D is the ingested dose (CFU); α and β are the model parameters. Given infection, Black et al. (1988) provided data on the likelihood of disease. On the basis that 29 out of 89 infected persons became ill, it is hypothesized that Pill|inf = 0.33 is a straightforward model for the likelihood of sickness given infection. The equation for probability of illness is:

$$P_{ill} = P_{inf} \times P_{ill|inf} \tag{8}$$

The risk of infection per serving of chicken was then calculated by multiplying the chance of infection by the seasonally retail prevalence (Pang et al., 2017).

8.2.10 "What-if" scenarios

The best- and worst-case alternative scenarios for the basic QMRA model were analyzed, and the predicted total number of campylobacteriosis cases from each scenario was compared to the result from the baseline model. The effect of thawing methods (refrigerator thawing, running water thawing, microwave thawing, and ambient room temperature thawing) was considered by running simulations where only one thawing method was applied. For each thawing method, the temperature and time distribution were obtained from either literature or expert opinion (Table 8.1).

8.2.11 Risk modeling

Using @Risk software (Version 7.6.1; Palisade, Ithaca, NY), all distribution fitting, correlation matrix application, and simulations were conducted. Where appropriate, *Campylobacter* concentrations were converted to decimal $\log_{10}$ values. All Monte Carlo simulations were conducted with a total of 100,000 iterations with Latin hypercube distribution sampling. To serve as the seed for all simulations, a random number between 1 and 100 (chosen number: 28) was selected at random. Using the RiskSimtable function in @Risk, uncertainty assessments were conducted. The correlation coefficients of Spearman were utilized in sensitivity analysis to examine the influence of distribution factors on output variables.

8.3 Results and discussion

8.3.1 Seasonal effect on presence of *Campylobacter* in chicken

Willis and Murray (1997) and Hinton et al. (2004) provided data on the prevalence of *Campylobacter* in chicken meat on a monthly basis. The seasonal prevalence was computed using the mean and is shown in Table 8.2. Similarly, monthly *Campylobacter* concentrations were collected, and the seasonal concentration was computed (Nannapaneni et al., 2009). *Campylobacter* prevalence was stable throughout the spring, summer, and fall months (0.53 to 0.59) but was lower during the winter months (0.26). (Table 8.2). With minimal change, *Campylobacter* concentrations were lowest during summer (1.74 log CFU/carcass) and highest in Fall (2.35 log CFU/carcass). Berrang et al. (2017) examined *Campylobacter* concentrations in cecal samples obtained from a Georgia processing plant. The data demonstrated a similar prevalence pattern. The prevalence of *Campylobacter* was greater in the warmer months (March to November) (0.53 to 0.64) than in the cooler months (December to January) (0.46). In Alabama, 0.41 percent of skinless chicken breasts were contaminated with *Campylobacter*

(Williams and Oyarzabal, 2012). Additionally, seasonal patterns were identified in other nations. There was a significant seasonal pattern in retail chicken meat over the summer and Fall months in Denmark (Boysen et al., 2011) and Wales (Louis et al., 2005). Lynch et al. (2022) found a considerably higher *Campylobacter* prevalence on chicken ceca samples in July (0.85 against other months in Ireland) compared to other months. Garcia-Sanchez et al. (2020) determined that spring and autumn are the most important seasonal variables for *Campylobacter* prevalence on a Spanish farm.

8.3.2 Growth rates

During the literature search, few data on *Campylobacter* growth on chicken meat (chicken parts, ground chicken, or chicken skin) were found. Consequently, the primary growth rates of *Campylobacter* were determined between 37 and 42°C. The parameters b (0.04673) and $T_{\min}$ (31.96 °C) were determined by fitting the secondary Ratkowsky model to growth rates, yielding an R^2 of 0.603. Due to the restricted amount of accessible data points, point estimates were utilized instead of distribution in the QMRA model. Furthermore, the observed minimum growth temperature (31 °C) of *Campylobacter* was determined and included into the QMRA model (Hazeleger et al., 1998; Park, 2002).

8.3.2 Effects of ambient temperature

As established by Golden and Mishra (2021), temperature variation was taken into account during shipping and thawing. Newton's law of heating was applied to chicken flesh in order to account for the amount of time it takes for chicken to achieve its ambient temperature when placed in a warmer environment. Newton's heating constant B had an average value of 2.26 h^{-1} (standard deviation: 0.54 h^{-1}). This number helps to estimate the surface temperature of chicken after a certain amount of time at a specified ambient temperature. This is crucial for calculating how much pathogens proliferate on the trip from a store's refrigerated storage to a consumer's house, since the product's temperature often rises during this period (Mercier et al., 2017). Moreover, according to a study, the temperature of fresh meat left in a vehicle trunk for two hours in the summer (average ambient temperature of 32.6 °C) reached 34.4 °C (Kim et al., 2013). During transportation, meat products may readily enter the danger zone for microbial development if exposed to high ambient temperatures. The average travel time from grocery shops to customers' homes was 1.2 hours, whereas the USDA Food Safety and Inspection Service recommended that perishable items be refrigerated within two hours (USDA/FSIS, 2008). When the outside temperature exceeds 32.2 °C, perishable items must be placed in the refrigerator within one hour.

8.3.4 Baseline QMRA model

For seasonal effect, the risk of infection and illness per serving were shown in Table 8.3. The mean risk of infection per serving was 1.31×10^{-3}, 1.57×10^{-3}, 1.45×10^{-3}, and 7.01×10^{-4} for spring, summer, fall, and winter, respectively. These results reflect the seasonal trend seen in retail *Campylobacter* prevalence, where winter season showed lower value compared to warmer seasons. To calculate the number of illnesses caused by each season in the United States, the total number of servings for each season was calculated using public data and the reference amount commonly eaten (RACC) per eating occasion for chicken meat of 85 g (9 CFR 381.412) (CLS, 2020). Due to the absence of information about the minimum and maximum serving sizes, only the RACC value of 85 g was employed in this research. This resulted in an estimated total of 17,247,755,827 seasonal meals of chicken meat prepared at home. The estimated average number of infections and illnesses in spring caused by consuming in-home prepared chicken in the baseline QMRA model were 22,571,609 (median 13,050) and 7,448,639 (median 4,307), respectively (Table 8.4). The cumulative distribution of number of infections and illnesses by season is shown in Figure 8.2 and 8.3. Despite the influence of outlier simulation results on the average, these results offer an estimate of the number of infections throughout the population and serves to demonstrate the uncertainty around the estimate, while the median helps to illustrate the distribution of simulation results.

The baseline QMRA model predicted an average of approximately 86,657,118 cases (median: 50,493) of campylobacteriosis infection annually. The estimated number of *Campylobacter* infections are 2.4 million every year (IBIS, 2021), which is lower than our prediction. This may be due to the fact that campylobacteriosis is largely underreported (Wagenaar et al., 2013). The predicted mean number of illnesses annually was 28,596,849 (median 16,663) from baseline QMRA model. Between 2009 and 2010, the U.S. National Outbreak Reporting System received reports of 56 confirmed and 13 suspected outbreaks, among which 1,550 illnesses and 52 hospitalizations were documented (Hall et al., 2013). Furthermore, based on outbreak data from 1998 to 2008, it was projected that 845,024 cases of campylobacteriosis occurred year in the United States, resulting in 8,463 hospitalizations and 76 fatalities (Batz et al., 2012). From 1996 to 2012, the U.S. Food-Borne Diseases Active Surveillance Network reported an annual incidence of *Campylobacter* infection of 14.3 per 100,000 people (Gilliss et al., 2013).

An important aspect of campylobacteriosis case distribution is the considerable seasonality and age-related fluctuation in incidence rates (Engberg, 2006; Hall et al., 2006; Nylen et al., 2002). Poultry is of special relevance to the overall epidemiology of campylobacteriosis, since they are often infected and may shed the germs in extremely large numbers (Bull et al., 2006; Park, 2002). Following the slaughtering process, contamination of poultry meat is common, and several case-control studies have linked the handling or ingestion of chicken meat to human illnesses (Engberg, 2006). Season is often connected with temperature, and season may also impact campylobacteriosis risk by seasonal differences in human activity, food supply, or changes in natural ecosystems. Higher temperatures may lead to an increase in the incidence of *Campylobacter* in animal populations or water, or to an increase in temperature abuse during food transit, storage, or handling (Boysen et al., 2011). Seasonality may have an effect independent of temperature, since human activities that facilitate exposure to *Campylobacter* fluctuate with the seasons. Seasonal variations in travel, swimming in untreated water, playground use, and direct contact with cattle, other animals, and flies are all related with higher risks of campylobacteriosis.

8.3.5 Uncertainty analysis

The numbers of infections and illnesses based on thawing methods are shown in Table 8.5. Thawing chicken meat under ambient room temperature significantly increases the total number of infections. Due to improper thawing, packaging of meat with other ready-to-eat foods, and poor handling of food contact materials, there was a high risk of cross contamination. Mkhungo et al. (2018) reported that 28% of people left their meat product on kitchen counter to thaw. Thawing takes more time than freezing, and when ambient air or running water is used, some parts of the raw meat are exposed to temperatures that are conducive for microbial growth (Leygonie et al., 2012). Also, the water that comes out of thawing meat is full of nutrients that could help bacteria grow. It does not seem that the amount of live bacteria present in meat is reduced by either the freezing or thawing process. The process of freezing, on the other hand, causes bacteria to enter a state of dormancy, which effectively puts an end to microbial deterioration. During the thawing process, unfortunately, they recover their activity. As a result, ambient room temperature thawing for meat products raises a huge food safety risk for consumers.

Table 5 summarizes the statistics for annual number of infections calculated during the uncertainty analyses. High *Campylobacter* prevalence showed higher mean number of infections (92,231,575) but the difference with low and medium *Campylobacter* prevalence was not significant. Whether to wash hands after handling raw chicken showed great difference in the number of infections. The median cases of always hand washing were 26,583 compared to that of never wash hands was 11,308,686. Similarly, always using different utensils when cooking chicken products showed median number of infections of 42,190 whereas the number of infections for never use different utensils was 2,694,612. Our results suggest that the cross-contamination during handling and cooking chicken meat showed more significant impact on the risk of campylobacteriosis than the initial prevalence of chicken products. The exposure assessment reveals that cross-contamination is the primary cause of bacteria exposure via food produced in kitchens (Pouillot et al., 2012). The author also conclude that cross-contamination appears to be a greater concern than bacterial development, even when products are held at high ambient temperatures. Kusumaningrum et al. (2004) examined unwashed surfaces as a cross-contamination factor during the preparation of chicken salad using ready-to-eat (RTE) ingredients. On average, 26% of consumers did not wash surfaces while preparing raw and cooked foods or ready-to-eat foods. Furthermore, cross-contamination from chicken to other ingredients via surface may happen. In addition, Lopez et al. (2015) found that using disinfectant wipes on kitchen surfaces during preparation chicken meat could effectively reduce the risk of *Campylobacter* infections.

8.3.6 Sensitivity analysis

Cross-contamination events (hands wash reduction; whether the hands are washed; and transfer from hands to cooked chicken) were the three most significant QMRA variables for predicting total *Campylobacter* risk of infection per serving, followed by *Campylobacter* concentration at purchase and transfer rate from raw chicken to hands (Fig. 8.4). As we expected, the frequency of washing hands was the most significant factor in reducing the risk. While this may be seen as a method to lower the risk of illness due to the intake of chicken, another concern that should be addressed is the development of bacterial antimicrobial resistance to compounds contained in antimicrobial soaps, such as triclosan (Yu et al., 2010). The third most important risk factor identified in the present model is handling cooked chicken with raw meat-contaminated hands. In a 2008 study, between 73% and 100% of subjects who claimed to have washed their hands after handling raw chicken were found to have *Campylobacter jejuni* on their hands (de Jong et al., 2008). Similar outcomes were also observed that even when hands are well cleansed, large amounts of bacteria might remain (Montville et al., 2002). Moreover, a recent survey revealed that just 39.6% of customers properly cleansed their hands after handling raw chicken breast (Maughan et al., 2016). These findings and observations indicate that there is still a significant need for improvement in consumer education on the safety of chicken products. Many of the people cleaned their hands by washing or rinsing them after handling the raw chicken items; however, they did not wash their hands until after they had contaminated other parts of the kitchen by touching things such as spices, utensils, or cooking surfaces. Additionally, Signorini et al. (2013) found the frequency of washing cutting board and hands were the second and forth important factors in human campylobacteriosis risk in a risk assessment carried out in

Argentina. The author also reported that during food preparation, the risk of human campylobacteriosis was 1.47 times larger for those who did not wash their hands.

This QMRA model was developed to represent the existing knowledge and practices of the retail-to-consumer supply chain for broiler meat. Consequently, a number of assumptions were included into the model and knowledge gaps were found. First, there were few data on the development of *Campylobacter* on chicken meat. More information will assist explain the growth characteristics and behavior of *Campylobacter* on chicken more precisely. It was presumed that customers did not transport meat from the grocery to their homes in a chilled state. It is probable that other items purchased with chicken meat might influence the temperature of the chicken during transportation, however no information is available to address this issue. In addition, information on storage and display times in U.S. grocery shops may be required. Next, assumptions have to be established about thawing timeframes for each of the evaluated thawing procedures in order to match the behavior that United States customers exhibit most often. Data on freezing technique trends were accessible, but information on the actual operations carried out throughout these methods was missing, necessitating reliance on USDA recommendations and internal expert opinion (USDA, 2013). In the absence of relevant *Campylobacter* transfer rate data, it was also assumed that *Campylobacter* transfer rates are comparable to those of the surrogates used in the included cross-contamination investigations. Other identified forms of cross-contamination events, like chicken washing and cross-contamination from other food products, were not included in the QMRA model (USDA/ERS, 2019). For chicken washing, statistics on the transfer rate of chicken to different kitchen surfaces were unavailable. Cross-contamination from other foods was not considered since the present model was only focused on estimating the number of yearly illnesses caused by broiler meat. In addition, it was assumed that each customer ingested just one serving of chicken meat at a time, and their infection risk was calculated based on this single serving. In reality, people may take many portions in a single sitting, but are only infected once. Finally, this model was constructed with several varieties of broiler meat in mind, including chicken parts and chicken meal. While factors such as contamination, package size, consumption, and portion size may vary with different types of broiler meat, many of the parameters used in the QMRA model included data from numerous types of broiler meat; thus, a model with a broad scope was developed to estimate the risk posed by these various types of chicken meat. When further data becomes available, this model may be modified in the future to concentrate on a certain variety of chicken meat.

8.4 Conclusions

To conclude, the current QMRA model predicts the number of seasonal cases of campylobacteriosis caused by consuming chicken meat processed at home in the United States. There was a seasonal influence on the risk of infection per serving, with the risk of *Campylobacter* infection in chicken being lower during the winter months. Similarly, the frequency of infections and diseases was less during the winter than during other seasons. Comparing room-temperature thawing to alternative thawing procedures in a "what-if" scenario, the number of infections was much greater for the room-temperature thawing method. According to the results of the sensitivity analysis, the hand-washing, the transfer rate from hands to cooked chicken, and whether the hands are washed are the three most influential variables on the overall number of infections and illnesses. The model shows a framework for chicken consumption, from retail to preparation and consumption at home. It also points out research needs to make the predictions more accurate, as well as ways to reduce the risk of salmonellosis in the United States caused by eating chicken meat.

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Table 8.1 Description of quantitative microbial risk assessment parameters in the baseline model

Variable	Cell	Distribution, value, or formula	Unit	Source
Growth parameter				
Growth model, b	B4	= 0.04673	No unit	(Blankenship, 1982; Nicorescu and Crivineanu, 2009; Solow et al., 2003)
Growth model, T_{min}	B5	= 31.96	°C	(Blankenship, 1982; Nicorescu and Crivineanu, 2009; Solow et al., 2003)
Observed T_{min}	B6	= 31	°C	(Hazeleger et al., 1998; Park, 2002)
Newton heating constant, B	B7	= 2.026	h^{-1}	(Golden and Mishra, 2021)
Retail				
Retail *Campylobacter* prevalence, Spring	B9	= RiskPert(0.41877,0.48933,0.48933)	Proportion	(Hinton et al., 2004; Willis and Murray, 1997)
Retail *Campylobacter* prevalence, Summer	B10	= RiskTriang(0.546,0.546,0.608227)	Proportion	(Hinton et al., 2004; Willis and Murray, 1997)
Retail *Campylobacter* prevalence, Fall	B11	= RiskUniform(0.507695,0.546205)	Proportion	(Hinton et al., 2004; Willis and Murray, 1997)
Retail *Campylobacter* prevalence, Winter	B12	= RiskUniform(0.24443,0.268024)	Proportion	(Hinton et al., 2004; Willis and Murray, 1997)
Campylobacter concentration, if positive at purchase	B13	= RiskWeibull(2.5448,1.9265,RiskShift(-1.4281))	log CFU/g	(USDA, 2012)
Retail cold room storage time	B14	= RiskExpon(0.58736,RiskShift(0.00027443))*24	h	(Derens et al., 2006)
Retail cold room storage temperature	B15	= RiskNormal(3.3188,1.7533)	°C	(Derens et al., 2006)
Retail display storage time	B16	= RiskExpon(0.22461,RiskShift(-0.0000889766))*24	h	(Derens et al., 2006)
Retail display storage temperature	B17	= RiskNormal(3.2321,1.3117)	°C	(Derens et al., 2006)

Growth rate during retail storage	B18	= IF(B15<B6,0,(B4*(B15-B5))^2)+IF(B17<B6,0,(B4*(B17-B5))^2)	log CFU/h	Calculated
Change during retail storage	B19	= B18*(B14+B16)	log CFU/g	Calculated
Concentration at point of purchase	B20	= RiskOutput()+IF((B19+B13)>5,5,B19+B13)	log CFU/g	Calculated
Transportation				
Ambient temperature during transportation, Spring	B22	= RiskPert(1.7239,21.039,35.837)	°C	(CR, 2022)
Ambient temperature during transportation, Summer	B23	= RiskPert(17.826,30.476,42.062)	°C	(CR, 2022)
Ambient temperature during transportation, Fall	B24	= RiskPert(3.9191,21.889,39.226)	°C	(CR, 2022)
Ambient temperature during transportation, Winter	B25	= RiskPert(-6.2993,10.263,29.747)	°C	(CR, 2022)
Transportation time	B26	= RiskLoglogistic(0.0063772,1.0915,4.6212,RiskTruncate(0.3,18.45))	h	(Ecosure, 2008)
Transportation growth rate, Spring	B27	= IF(B22<B6,0,(B4*((B22-(EXP(-B7*B26)*(B22-B17)))-B5))^2)	log CFU/h	Calculated
Transportation growth rate, Summer	B28	= IF(B23<B6,0,(B4*((B23-(EXP(-B7*B26)*(B23-B17)))-B5))^2)	log CFU/h	Calculated
Transportation growth rate, Fall	B29	= IF(B24<B6,0,(B4*((B24-(EXP(-B7*B26)*(B24-B17)))-B5))^2)	log CFU/h	Calculated
Transportation growth rate, Winter	B30	= IF(B25<B6,0,(B4*((B25-(EXP(-B7*B26)*(B25-B17)))-B5))^2)	log CFU/h	Calculated
Change during transportation, Spring	B31	= B27*B26	log CFU/g	Calculated
Change during transportation, Summer	B32	= B28*B26	log CFU/g	Calculated

Change during transportation, Fall	B33	= B29*B26	log CFU/g	Calculated
Change during transportation, Winter	B34	= B30*B26	log CFU/g	Calculated
Concentration after transportation, Spring	B35	= B31+B20	log CFU/g	Calculated
Concentration after transportation, Summer	B36	= B32+B20	log CFU/g	Calculated
Concentration after transportation, Fall	B37	= B33+B20	log CFU/g	Calculated
Concentration after transportation, Winter	B38	= B34+B20	log CFU/g	Calculated
Home storage				
Does chicken get frozen?	B40	= RiskBernoulli(0.4)	No unit	(Mazengia et al., 2015)
If frozen:				
Time until frozen	B42	= RiskBetaGeneral(0.0067951,0.59992,0,2)	h	(Mazengia et al., 2015)
Ambient room temperature	B43	= RiskNormal(22.3107,5.8722,RiskTruncate(15,30))	℃	(Booten et al., 2017)
Growth rate before products were put in freezer	B44	= IF(B43<B6,0,(B4*(B43-B5))^2)	log CFU/h	Calculated
Change before frozen	B45	= B44*B43	log CFU/g	Calculated
Concentration before frozen, Spring	B46	= B45+B35	log CFU/g	Calculated
Concentration before frozen, Summer	B47	= B45+B36	log CFU/g	Calculated
Concentration before frozen, Fall	B48	= B45+B37	log CFU/g	Calculated
Concentration before frozen, Winter	B49	= B45+B38	log CFU/g	Calculated
Home refrigerator temperature	B50	= RiskLaplace(4.4444,2.5231)	℃	(Bruhn, 2014)
Home freezer temperature	B51	= RiskNormal(-9.275,5.2857,RiskTruncate(-25,0))	℃	(Byrd-Bredbenner et al., 2007)

Thawing method	B52	= RiskDiscrete({1,2,3,4},{0.48,0.14,0.24,0.14})	No unit	(Mazengia et al., 2015)
If thaw method = 1:				
Thaw time	B54	= RiskTriang(2,24,72)	h	
Growth rate during refrigerated thawing	B55	= IF(B50<B6,0,(B4*((B50-(EXP(-B7*B54)*(B50-B51)))-B5))^2)	log CFU/h	Calculated
Change during refrigerated thawing	B56	= IF(B52=1,B54*B55,0)	log CFU/g	Calculated
If thaw method = 2:				
Running water temperature	B58	= RiskPert(14,22.9,30)	°C	(Evison and Sunna, 2001)
Thaw time	B59	= RiskTriang(0.25,1,2)		
Growth rate during running water thawing	B60	= (B4*((B58-(EXP(-B7*B59)*(B58-B51)))-B5))^2	log CFU/h	Calculated
Change during running water thawing	B61	= IF(B52=2,B59*B60,0)	log CFU/g	Calculated
If thaw method = 3:				
Temperature of meat during microwave thawing	B63	= RiskPert(-8,-4,8)	°C	(Taher and Farid, 2001)
Thaw time	B64	= RiskUniform(8,20)/60	h	
Growth rate during microwave thawing	B65	= IF(B63<B6,0,(B4*((B63-(EXP(-B7*B64)*(B63-B51)))-B5))^2)	log CFU/h	Calculated
Change during microwave thawing	B66	= IF(B52=3,B64*B65,0)	log CFU/g	Calculated
If thaw method = 4:				
Ambient room temperature	B68	= RiskNormal(22.3107,5.8722,RiskTruncate(15,30))	°C	(Booten et al., 2017)
Thaw time	B69	= RiskUniform(1,10)	h	
Growth during room temperature thawing	B70	= (B4*((B68-(EXP(-B7*B69)*(B68-B51)))-B5))^2	log CFU/h	Calculated
Change during room temperature thawing	B71	= IF(B52=4,B69*B70,0)	log CFU/g	Calculated
Concentration after thawing, Spring	B72	= IF(B40=1,B46+B56+B61+B66+B71,0)	log CFU/g	Calculated
Concentration after thawing, Summer	B73	= IF(B40=1,B47+B56+B61+B66+B71,0)	log CFU/g	Calculated

Concentration after thawing, Fall	B74	= IF(B40=1,B48+B56+B61+B66+B71,0)	log CFU/g	Calculated
Concentration after thawing, Winter	B75	= IF(B40=1,B49+B56+B61+B66+B71,0)	log CFU/g	Calculated
If not frozen:				
Refrigerator storage time	B78	= RiskPareto(3.4887,2,RiskTruncate(0,5))*24	h	(Mazengia et al., 2015)
Growth rate during refrigerated storage	B79	= IF(B50<B6,0,(B4*(B50-B5))^2)	log CFU/h	Calculated
Change during storage	B80	= B77*B78	log CFU/g	Calculated
Concentration after storage, Spring	B81	= IF(B40=1,0,B35+B79)	log CFU/g	Calculated
Concentration after storage, Summer	B82	= IF(B40=1,0,B36+B79)	log CFU/g	Calculated
Concentration after storage, Fall	B83	= IF(B40=1,0,B37+B79)	log CFU/g	Calculated
Concentration after storage, Winter	B84	= IF(B40=1,0,B38+B79)	log CFU/g	Calculated
Concentration before preparation, Spring	B85	= B72+B80	log CFU/g	Calculated
Concentration before preparation, Summer	B86	= B73+B81	log CFU/g	Calculated
Concentration before preparation, Fall	B87	= B74+B82	log CFU/g	Calculated
Concentration before preparation, Winter	B88	= B75+B83	log CFU/g	Calculated
Preparation				
Raw chicken handling:				
Transfer rate from raw chicken to hands	B90	= RiskLognorm(0.15555,1.0547, RiskShift(0.00058696),RiskTruncate(0,1))	Proportion	(Chen et al., 2001; Luber et al., 2006; Verhoeff-Bakkenes et al., 2008)
Concentration on hands after handling, Spring	B91	= LOG10(B90*(10^B85))	log CFU/g	Calculated
Concentration on hands after handling, Summer	B92	= LOG10(B90*(10^B86))	log CFU/g	Calculated

Concentration on hands after handling, Fall	B93	= LOG10(B90*(10^B87))	log CFU/g	Calculated
Concentration on hands after handling, Winter	B94	= LOG10(B90*(10^B88))	log CFU/g	Calculated
Concentration left on chicken, Spring	B95	= IF(10^B85-10^B91=0,0,LOG10(10^B85-10^B91))	log CFU/g	Calculated
Concentration left on chicken, Summer	B96	= IF(10^B86-10^B92=0,0,LOG10(10^B86-10^B92))	log CFU/g	Calculated
Concentration left on chicken, Fall	B97	= IF(10^B87-10^B93=0,0,LOG10(10^B87-10^B93))	log CFU/g	Calculated
Concentration left on chicken, Winter	B98	= IF(10^B88-10^B94=0,0,LOG10(10^B88-10^B94))	log CFU/g	Calculated
Transfer rate from raw chicken to utensils	B99	= RiskLognorm(0.0064271,0.28575,RiskShift(0.00000124688),RiskTruncate(0,1))	Proportion	(Chen et al., 2001; Luber et al., 2006; Verhoeff-Bakkenes et al., 2008)
Concentration on utensils after handling, Spring	B100	= LOG10((10^B95)*B99)	log CFU/g	Calculated
Concentration on utensils after handling, Summer	B101	= LOG10((10^B96)*B99)	log CFU/g	Calculated
Concentration on utensils after handling, Fall	B102	= LOG10((10^B97)*B99)	log CFU/g	Calculated
Concentration on utensils after handling, Winter	B103	= LOG10((10^B98)*B99)	log CFU/g	Calculated
Concentration on chicken, Spring	B104	= LOG10(10^B95-10^B100)	log CFU/g	Calculated
Concentration on chicken, Summer	B105	= LOG10(10^B96-10^B101)	log CFU/g	Calculated
Concentration on chicken, Fall	B106	= LOG10(10^B97-10^B102)	log CFU/g	Calculated
Concentration on chicken, Winter	B107	= LOG10(10^B98-10^B103)	log CFU/g	Calculated
Cooking:				

Is chicken undercooked?	B109	= RiskBernoulli(0.399)	No unit	(Ecosure, 2008)
Cooking time	B110	= RiskPert(15,30,45,RiskCorrmat(NewMatrix1,1))	Min	(Oscar, 2004)
Cooking temperature	B111	= RiskPert(38.244,82.305,100.48, RiskTruncate(38.244, 73.9),RiskCorrmat(NewMatrix1,2))	°C	(Bruhn, 2014)
D-value	B112	= 10^(-0.96-(B111-70)/12.3)	Min	(van Asselt and Zwietering, 2006)
Change during undercooking	B113	= B110/B112	log CFU/g	Calculated
Concentration after undercooking, Spring	B114	= B104-B113	log CFU/g	Calculated
Concentration after undercooking, Summer	B115	= B105-B113	log CFU/g	Calculated
Concentration after undercooking, Fall	B116	= B106-B113	log CFU/g	Calculated
Concentration after undercooking, Winter	B117	= B107-B113	log CFU/g	Calculated
Cooked product handling:				
Are hands washed?	B119	= RiskBernoulli(0.883)	No unit	(Kosa et al., 2015)
Hand washing reduction	B120	= RiskNormal(2.7163,1.2661,RiskTruncate(0.34,5.29))	log CFU/g	(Chen et al., 2001)
Concentration on hands after washing, Spring	B121	= B91-B120	log CFU/g	Calculated
Concentration on hands after washing, Summer	B122	= B92-B120	log CFU/g	Calculated
Concentration on hands after washing, Fall	B123	= B93-B120	log CFU/g	Calculated
Concentration on hands after washing, Winter	B124	= B94-B120	log CFU/g	Calculated

Transfer rate to cooked chicken by hands	B125	= RiskLevy(-0.0003382,0.0019097,RiskTruncate(0,1))	Proportion	(Chen et al., 2001; Luber et al., 2006)
Concentration after handling cooked chicken with hands, Spring	B126	= LOG10(IF(B119=0,(10^B91)*B125,(10^B121)*B125)+IF(B109=0,0, 10^B114))	log CFU/g	Calculated
Concentration after handling cooked chicken with hands, Summer	B127	= LOG10(IF(B119=0,(10^B92)*B125,(10^B122)*B125)+IF(B109=0,0, 10^B115))	log CFU/g	Calculated
Concentration after handling cooked chicken with hands, Fall	B128	= LOG10(IF(B119=0,(10^B93)*B125,(10^B123)*B125)+IF(B109=0,0, 10^B116))	log CFU/g	Calculated
Concentration after handling cooked chicken with hands, Winter	B129	= LOG10(IF(B119=0,(10^B94)*B125,(10^B124)*B125)+IF(B109=0,0, 10^B117))	log CFU/g	Calculated
Are different dishes or utensils used?	B130	= RiskBernoulli(0.959)	No unit	(Kosa et al., 2015)
Transfer rate to cooked chicken by dirty utensils	B131	= RiskExpon(0.12217,RiskShift(-0.00041787),RiskTruncate(0,1))	Proportion	(Chen et al., 2001)
Final concentration, Spring	B132	= LOG10(10^B126+IF(B130=0,B131*(10^B100),0))	log CFU/g	Calculated
Final concentration, Summer	B133	= LOG10(10^B127+IF(B130=0,B131*(10^B101),0))	log CFU/g	Calculated
Final concentration, Fall	B134	= LOG10(10^B128+IF(B130=0,B131*(10^B102),0))	log CFU/g	Calculated
Final concentration, Winter	B135	= LOG10(10^B129+IF(B130=0,B131*(10^B103),0))	log CFU/g	Calculated
Dose-response and infection				
Serving size	B137	= 85	g	9 CFR §381.412
Concentration per serving, Spring	B138	= (10^B132)*B137	CFU	Calculated
Concentration per serving, Summer	B139	= (10^B133)*B137	CFU	Calculated
Concentration per serving, Fall	B140	= (10^B134)*B137	CFU	Calculated
Concentration per serving, Winter	B141	= (10^B135)*B137	CFU	Calculated

Dose response infection, alpha	B142	= 0.145	No unit	(Teunis and Havelaar, 2000)
Dose response infection, beta	B143	= 7.59	No unit	(Teunis and Havelaar, 2000)
Probability of infection, Spring	B144	= 1-(1+(B138/B143))^-B142	No unit	Calculated
Probability of infection, Summer	B145	= 1-(1+(B139/B143))^-B142	No unit	Calculated
Probability of infection, Fall	B146	= 1-(1+(B140/B143))^-B142	No unit	Calculated
Probability of infection, Winter	B147	= 1-(1+(B141/B143))^-B142	CFU	Calculated
Probability of illness, Spring	B148	= B144*0.33	No unit	(Black et al., 1988; Havelaar et al., 2000; Nauta et al., 2007)
Probability of illness, Summer	B149	= B145*0.33	No unit	(Black et al., 1988; Havelaar et al., 2000; Nauta et al., 2007)
Probability of illness, Fall	B150	= B146*0.33	No unit	(Black et al., 1988; Havelaar et al., 2000; Nauta et al., 2007)
Probability of illness, Winter	B151	= B147*0.33	No unit	(Black et al., 1988; Havelaar et al., 2000; Nauta et al., 2007)
Risk of infection per serving, Spring	B152	= B144*B9	No unit	Calculated
Risk of infection per serving, Summer	B153	= B145*B10	No unit	Calculated
Risk of infection per serving, Fall	B154	= B146*B11	No unit	Calculated
Risk of infection per serving, Winter	B155	= B147*B12	No unit	Calculated
Risk of illness per serving, Spring	B156	= B148*B9	No unit	Calculated
Risk of illness per serving, Summer	B157	= B149*B10	No unit	Calculated
Risk of illness per serving, Fall	B158	= B150*B11	No unit	Calculated

Risk of illness per serving, Winter	B159	= B151*B12	No unit	Calculated
Total per capita poultry availability per year	B160	= 43454.15	g	(USDA/ERS, 2019)
Total used in raw chicken preparation per year	B161	= 21727.075	g	(NCC, 2015)
U.S. population	B162	= 325186237	People	(USDA/ERS, 2019)
Number of consumers who purchased chicken from grocery/supermarket	B163	= 269904576.7	People	(NCC, 2014)
Consumed serving per person per season	B164	= 63.90	Serving	Calculated
No. of servings consumed per season in US	B165	= 17247755827	No unit	Calculated
No. of infections per season, Spring	B166	= B165*B152	No unit	Calculated
No. of infections per season, Summer	B167	= B165*B153	No unit	Calculated
No. of infections per season, Fall	B168	= B165*B154	No unit	Calculated
No. of infections per season, Winter	B169	= B165*B155	No unit	Calculated
No. of illness per season, Spring	B170	= B165*B156	No unit	Calculated
No. of illness per season, Summer	B171	= B165*B157	No unit	Calculated
No. of illness per season, Fall	B172	= B165*B158	No unit	Calculated
No. of illness per season, Winter	B173	= B165*B159	No unit	Calculated
Total number of infections per year	B174	= B166+B167+B168+B169	No unit	Calculated
Total number of illnesses per year	B175	= B170+B171+B172+B173	No unit	Calculated

Table 8.2 Seasonal trends of *Campylobacter* prevalence, concentrations, and outbreaks in chicken products

	Spring	Summer	Fall	Winter
Campylobacter prevalence (Average±SD)	0.59±0.32	0.56±0.48	0.53±0.41	0.26±0.32
Campylobacter concentration (log CFU/carcass) (Average±SD)	2.26±0.56	1.74±0.89	2.35±0.85	2.30±1.28
Campylobacter outbreaks	17	25	15	10

[a] Outbreaks data were extracted from the National Outbreak Reporting System (NORS) from 1998 to 2020 strictly relate to chicken and *Campylobacter*.

Table 8.3 Summary statistics of risk of infection and illness per season determined by QMRA baseline model

Seasonal effect	Risk of infection per serving				Risk of illness per serving			
	Mean	Median	25%	75%	Mean	Median	25%	75%
Spring	1.31×10^{-3}	7.57×10^{-7}	3.56×10^{-8}	1.85×10^{-5}	4.32×10^{-4}	2.50×10^{-7}	1.17×10^{-8}	6.09×10^{-6}
Summer	1.57×10^{-3}	9.22×10^{-7}	4.29×10^{-8}	2.24×10^{-5}	5.18×10^{-4}	3.04×10^{-7}	1.42×10^{-8}	7.40×10^{-6}
Fall	1.45×10^{-3}	8.40×10^{-7}	3.92×10^{-8}	2.05×10^{-5}	4.77×10^{-4}	2.77×10^{-7}	1.29×10^{-8}	6.75×10^{-6}
Winter	7.01×10^{-4}	4.06×10^{-7}	1.89×10^{-8}	9.91×10^{-6}	2.31×10^{-4}	1.34×10^{-7}	6.25×10^{-9}	3.27×10^{-6}

Table 8.4 Summary statistics of number of infections and illnesses per season

Seasonal effect	No. of infections per season				No. of illnesses per season			
	Mean	Median	25%	75%	Mean	Median	25%	75%
Spring	22,571,609	13,050	611	318,258	7,448,639	4,306	201	105,034
Summer	27,058,680	15,895	739	386,805	8,929,364	5,245	244	127,646
Fall	24,941,190	14,488	676	353,010	8,230,593	4,781	223	116,493
Winter	12,085,638	7,008	327	170,841	3,988,261	2,312	108	56,378

Table 8.5 Summary statistics for total number of infections annually of uncertainty analysis.

Scenario	No. of infections			
	Mean	Median	25%	75%
Baseline	86,657,118	50,493	2,355	1,228,846
Uncertainty, prevalence:				
Low	79,734,367	48,471	2,179	1,212,001
Medium	90,535,132	50,282	2,375	1,305,955
High	92,231,575	53,480	2,525	1,349,224
Thawing method:				
Refrigerator thawing	53,162,035	42,674	2,014	1,010,711
Running water thawing	68,289,580	61,462	2,887	1,487,499
Microwave thawing	53,007,197	42,047	2,018	1,014,576
Ambient room temperature thawing	286,663,540	122,142	4,714	3,933
Hand washing:				
Always wash hands	41,774,442	26,583	1,569	471,588
Never wash hands	429,585,788	11,308,668	1,386,150	100,598,358
Cleaning:				
Always use different utensils	83,552,680	42,190	2,046	1,027,979
Never use different utensils	213,628,883	2,694,612	312,481	25,216,122

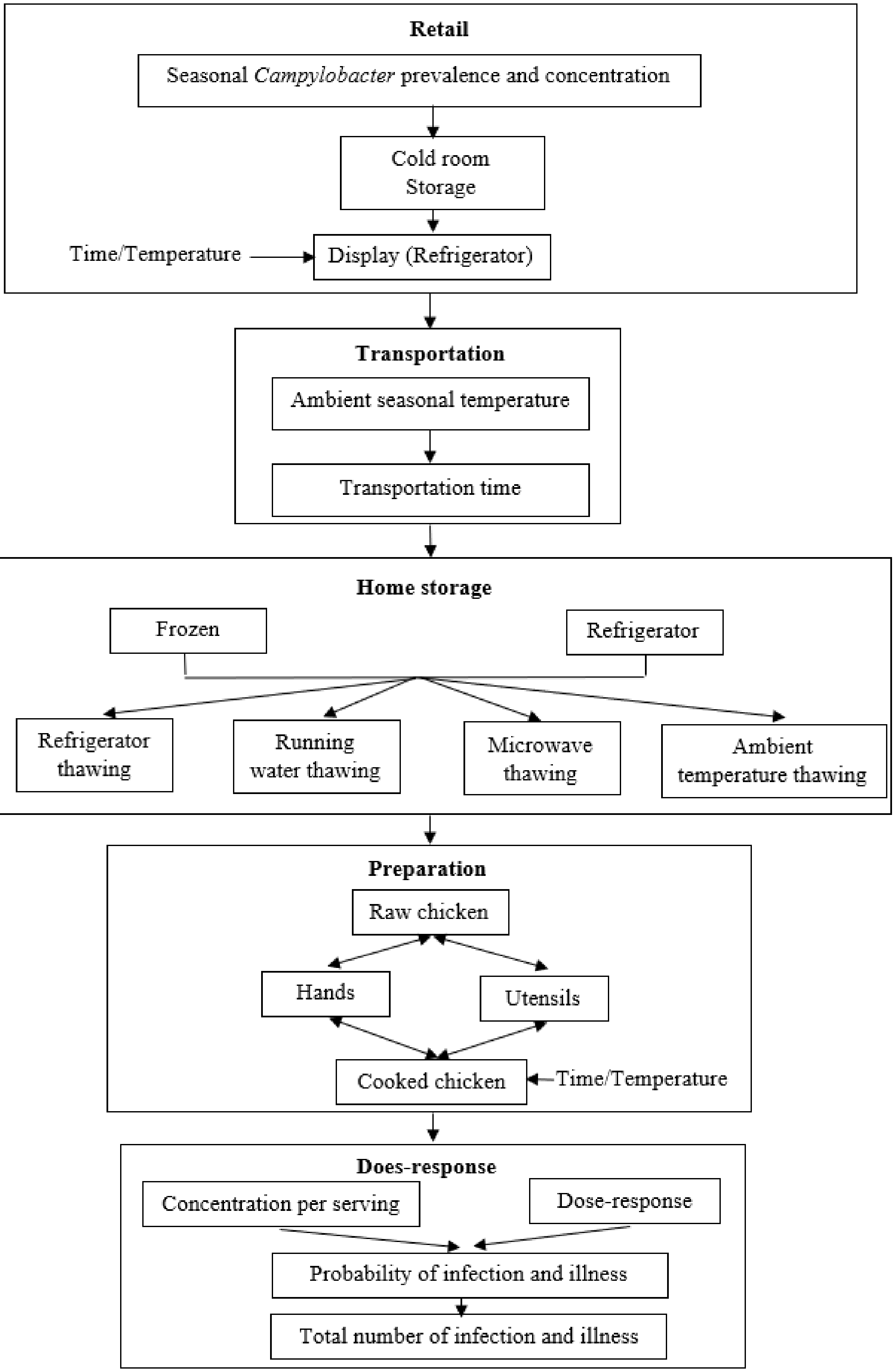

Figure 8.1 Overview of the quantitative microbial risk assessment model

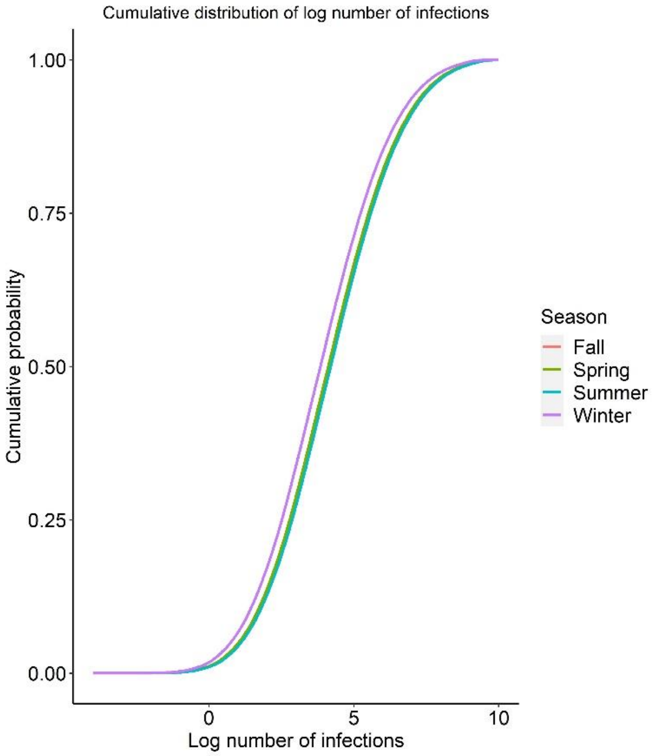

Figure 8.2 Cumulative distribution functions for log number of infections per season

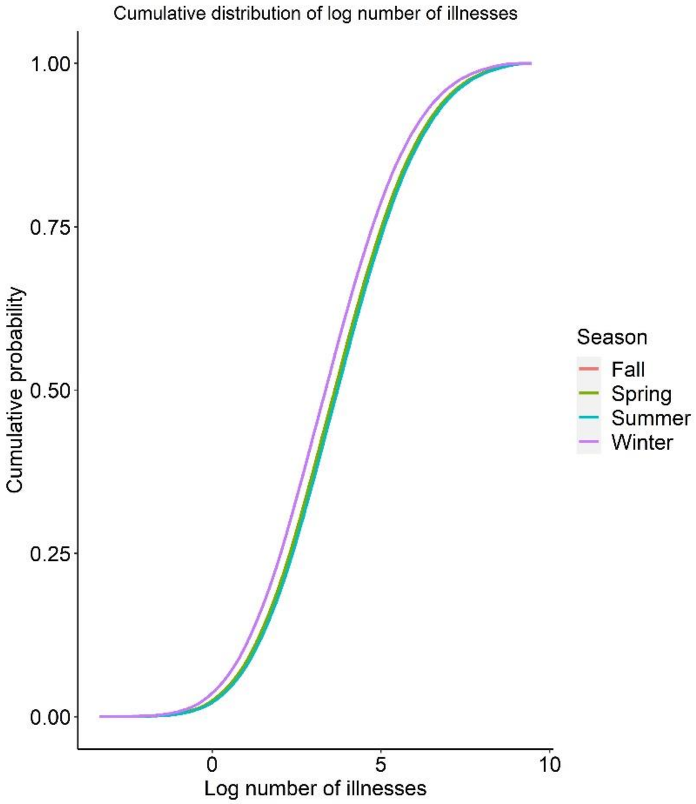

Figure 8.3 Cumulative distribution functions for log number of illnesses per season

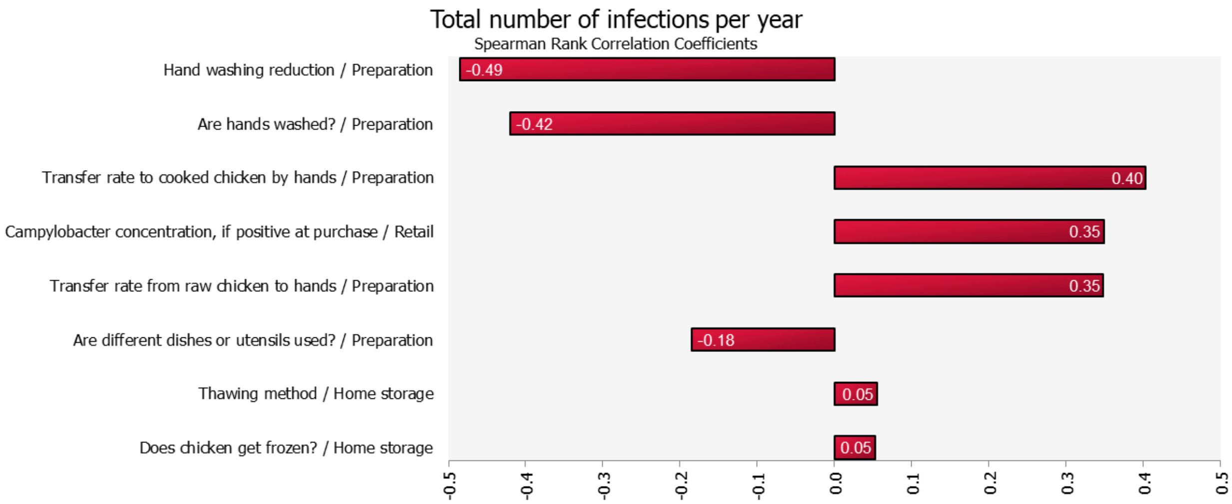

Figure 8.4 Spearman's correlation coefficients show the 8 most important model parameters for predicting the total number of infections in broiler meat

CHAPTER 9

SUMMARY AND CONCLUSIONS

In Chapter 3, the LASSO and RT models were used to estimate the concentration of generic *E. coli* in preharvest and postharvest samples from pastured poultry farms. Moreover, employing both machine learning models, the farm management practices that had the greatest influence on *E. coli* concentration were identified. Model performances were compared using root mean square error (RMSE). Using LASSO, the average number of birds and animal source were the two most influential factors on *E. coli* population in feces samples. The RT determined that animal source, brood diet, day of the year, flock age in days, and flock size were the best predictive factors for *E. coli* concentration. The RMSE (on a $\log_{10}$ scale) for feces samples was 0.974 under LASSO and 1.032 under RT. The prediction models offer practical and efficient methods for predicting *E. coli* population and identifying agricultural practice parameters that influence *E. coli* concentrations.

Since models to predict *E. coli* concentration were developed in Chapter 3, Chapter 4 explored the idea of employing *E. coli* as an indicator microbe to predict the presence or absence of foodborne pathogens. For each sample type, a logistic regression model for pathogen presence was created. *E. coli* was significantly linked with *Campylobacter*, *Salmonella*, and *Listeria* in soil and WCR-P samples. The connection between *E. coli* and *Listeria* in feces, soil, and ceca samples is generally weak. In addition, negative associations between *E. coli* and *Campylobacter* in feces samples were discovered. These models provide a realistic and valuable perspective on the interaction between *E. coli* and pathogens found in food. They also enable the prediction of foodborne infections based on the prevalence of *E. coli* in the farm-to-fork continuum for pastured poultry.

In Chapter 5, a random forest model was constructed to predict *Campylobacter* prevalence in feces and soil samples collected from pastured poultry farms and WCR-P samples collected from processing plants. The performance of models was evaluated by the area under curve AUC) from the receiver operating characteristics curve. The AUC values were 0.902, 0.894, and 0.864 for feces, soil, and WCR-P models, respectively. The animal source of feces was found as the most significant variable in the fecal model, while the soy content of the brood diet was recognized as the most significant variable in the soil model. For the WCR-P model, the average flock age had the most significant effect on the RF model. As mentioned in Chapter 3, the animal source was identified as the most important factor in predicting *E. coli* concentration as well as *Campylobacter*. These RF models may assist pastured poultry producers in implementing food safety management techniques to minimize *Campylobacter* prevalence on pastured poultry farms.

Chapter 6 and 7 were conducted to address the association between pastured poultry-related microbiomes and the presence and absence of foodborne pathogens. Samples were assessed for *Campylobacter* and *Salmonella* using selective media and molecularly using microbiome relative abundances via 16S rRNA amplicon sequencing. Results showed that cultural method showed higher *Campylobacter* prevalence in feces, soil, and ceca samples than microbiome method. In all sample types, the cultural method had a lower *Salmonella* prevalence than the microbiome method. In addition, Linear discriminant analysis (LDA) effect size (LEfSe) was used to identify taxa significantly enriched in *Campylobacter* and *Salmonella* positive samples or negative samples. For *Campylobacter*, the LEfSe comparison of two methods revealed that pre-harvest samples had better agreement than post-harvest samples. *Streptophyta*, *Cyanobacteria*, and Chloroplasts were significantly enriched in *Campylobacter* negative ceca, WCR-P, and WCR-F samples for post-harvest samples. For *Salmonella*, in terms of pre-harvest samples, *Planococcaceae* and *Bacillales* were identified as the common taxa enriched in *Salmonella* positive fecal samples whether assessed by cultural or microbiome methods, while taxa related to *Gamma-proteobacteria* were enriched in soil samples. *Pseudomonadales* and *Gamma-proteobacteria* taxa were enriched in *Salmonella* positive WCR-P samples, while taxa related to *Streptophyta*, *Cyanobacteria*, and Chloroplasts were greatly enriched in *Salmonella* negative WCR-P samples. These studies compared the cultural and microbiome approaches for identifying *Campylobacter* and *Salmonella* in poultry farms with pastured chickens. Both techniques employed common taxa to represent *Campylobacter* and *Salmonella*-positive or -negative samples, therefore giving information for future study on *Campylobacter* and *Salmonella*-related taxa.

A retail-to-consumption QMRA was undertaken in Chapter 8 to estimate the risk of *Campylobacter* to consumers in the United States from the preparation and consumption of broiler meat at home, as well as the risks associated with seasonality. During the summer, chicken meat was predicted to have a median risk of illness per serving of 9.22×10^{-7} and cause around 27,058,680 infections on average. During the winter months, the estimated median risk of infection per serving was 4.06×10^{-7}, resulting in an average of 12,085,638 illnesses. The analysis of sensitivity indicated that not washing hands before preparing food is the most significant risk factor. The "what-if" study revealed that thawing chicken at room temperature significantly increased the likelihood of contracting *Campylobacter* infections and becoming ill. The model of risk assessment gives information on the seasonal trend of broiler meat to public health. These findings will assist in identifying the most crucial measures to mitigate the food safety concerns associated with tainted chicken products.

CHAPTER 10

FUTURE STUDIES

This dissertation addressed knowledge gaps in foodborne pathogens' prevalence and generic *E. coli* concentration in pastured poultry farms, identified farm management practices important to pathogen prevalence and *E. coli* concentration, provided information on the relationship between pastured poultry-related microbiome and foodborne pathogens, and evaluated the seasonal pattern of *Campylobacter* contaminated poultry products prepared at home. However, further study is suggested in the areas listed.

In Chapter 3, numerous farm management-related parameters were chosen to demonstrate a substantial influence on the concentration of *E. coli* in different sample types. There is a need for well-defined experiments that control certain factors to confirm our results. For instance, the constituents of brood feed (pea, maize, and oat) were found as the most significant factors for predicting the concentration of *E. coli* in ceca samples. To explain and verify the conclusion that brood feed has a considerable influence on *E. coli* concentration in ceca, it is necessary to design the experimental plan that controls for feed and other variables (age, breed, sex, etc.).

Chapter 4 identified that use of soy in broiler feed had a significant effect on the prevalence of *Campylobacter* in soil samples. More research is required to comprehend the role and mechanism of soy in *Campylobacter* prevalence. Similarly, further study is required to validate the influence of parameters such as pasture housing, source of animal excrement, and average flock size on *Campylobacter* prevalence.

The fifth chapter examined the feasibility of using *E. coli* concentration as an indicator microorganism and forecasting the prevalence of foodborne pathogens in pastured poultry farms. Studies designed to find the relationship between *E. coli* and foodborne pathogens in other food systems, such as produce, are of tremendous interest and significance. In addition, it is of interest to investigate whether other types of indicating microbes (*Enterobacteriaceae* and *Pseudomonas*) may be utilized. In addition, the logistic regression model identified a statistically significant association between *E. coli* and foodborne pathogens. To scientifically comprehend the correlation's mechanisms, further study is required.

In Chapters 6 and 7, a comparison was made between the culture approach and the microbiome method for identifying *Campylobacter* and *Salmonella* in preharvest (feces and soil) and postharvest samples (ceca and whole carcass rinse). More data are required to demonstrate the microbiome method's accuracy. Future research may aid in determining the feasibility of using the microbiome technique in routine investigations. Additionally, utilizing LEfSe analysis, taxa representing *Campylobacter* or *Salmonella*-positive or -negative samples were identified. To comprehend the process and association between certain taxa and the existence of a pathogen, more study is required. In addition, postharvest samples exhibited a greater degree of fluctuation in LEfSe values than preharvest ones. There is a need for more research on the variation.

Chapter 8 presented a risk assessment of the seasonal impact of *Campylobacter*-contaminated chicken meat processed at home. Several information gaps were found when developing the risk assessment model. The growth parameters of *Campylobacter* at various temperatures, oxygen levels, light exposure, packaging methods, and chicken meat kinds (mutilated, whole carcass, and pieces) must first be identified. Second, seasonal data on *Campylobacter* prevalence and concentrations in chicken products and seasonal consumption trends in the United States are lacking. Furthermore, customer behavior data about thawing times for various thawing procedures are required. *Campylobacter* cross-contamination transfer rates from chicken to hands and utensils and *Campylobacter* transfer rates from hands and utensils to cooked chicken should be determined.

APPENDICES

Appendix A Complete coefficients of LASSO model for feces and soil samples in Chapter 3

Feces		Soil	
Selected variables (Level)	Coefficients	Selected variables (Level)	Coefficients
Intercept	6.966	Intercept	5.117
Ave Num Bird	-0.251	Years Farming	-0.021
Egg Source (D)	0.120	Egg Source (A)	0.113
Brood Bedding (WS)	0.100	Egg Source (F)	-0.411
Brood Feed (WC)	-0.184	Egg Source (E)	-0.412
Brood Feed (CSO)	0.105	Egg Source (D)	1.198
Brood Clean Frequency (AIAO)	0.171	Brood Feed (PCO)	-0.104
Brood Clean Frequency (DLM)	0.262	Brood Soy Free (Y)	0.771
Pasture Housing (CTF2)	-0.067	Brood Clean Frequency (AIAO)	0.433
Pasture Housing (CTFR)	0.155	Brood Clean Frequency (Weekly)	-1.160
Pasture Feed (WC)	-0.019	Brood Clean Frequency (Yearly)	0.026
Pasture Feed (CSO)	0.029	Ave Age to Pasture (4)	-1.625
Length Feed Restrix Process	0.004	Pasture Housing (CTFR)	0.0006
Flock Age Days	0.003	Frequency of Housing Moving (Daily)	0.0003
Breed (RR)	0.435	Pasture Feed (W)	0.954
Flock Size	-0.0005	Pasture Soy Free (Y)	-0.453
Animal Source (Cattle)	-1.253	Pasture Medicated (N)	-0.259
Animal Source (Layer)	0.618	Goats on Farm (Y)	0.547
Animal Source (Swine)	-0.665	Frequency Bird Handling (OIN)	-0.804
		Any ABX Use (Y)	-1.380
		Length Feed Restriction Process	0.020
		Day of year	-0.001
		Flock Age Days	0.012
		Breed (RR)	-1.110
		Animal Source (Cattle)	0.221
		Animal Source (Layer)	-0.007
		Animal Source (Swine)	-0.676

Appendix B Complete coefficients of LASSO model for WCR-P, WCR-F, and ceca samples in Chapter 3

WCR-P		WCR-F		Ceca	
Selected variables (Level)	Coefficients	Selected variables (Level)	Coefficients	Selected variables (Level)	Coefficients
Intercept	3.013	Intercept	1.350	Intercept	2.896
Avg Num Birds	-0.144	Years Farming	0.064	Avg Num Birds	0.690
Egg Source (A)	0.057	EggSourceD	-0.143	Ave Num Flocks	0.002
Egg Source (B)	0.768	EggSourceB	0.571	Egg Source (E)	-0.600
Brood Bedding (WS)	0.057	Brood Bedding (WS)	0.300	Egg Source (D)	0.832
Brood Soy Free (Y)	-0.510	Brood Soy Free (Y)	-0.321	Egg Source (B)	0.266
Brood Clean Frequency (AIAO)	0.111	Brood Clean Frequency (Weekly)	-0.124	Brood Feed (CSW)	0.299
Brood Clean Frequency (Weekly)	-0.124	Pasture Housing (CTF2)	-0.049	Brood Feed (CSO)	-0.245
Pasture Housing (CTFR)	0.006	Pasture Housing (CTFR)	1.055	Brood Feed (PCO)	0.419
Pasture Feed (W)	0.445	Pasture Feed (W)	-0.0007	Brood Soy Free (Y)	-0.646
Layers On Farm (Y)	0.124	Pasture Feed (CSW)	0.043	Avg Age to Pasture (4)	-0.872
Goats On Farm (Y)	-0.153	Water Source (Rain)	-0.131	Pasture Feed (W)	0.880
Any ABX Use (Y)	-0.331	Frequency Bird Handling (OIN)	-0.0007	Pasture Feed (PCO)	1.221
Scalder Temp (65 °C)	0.637	Any ABX Use (Y)	-0.0006	Layer On Farm (Y)	-0.313
Scalder Temp (82 °C)	-0.004	Processing Type (Plant)	-0.407	Goat On Farm (Y)	1.286
Rinse Water Chlor (NA)	0.901	Scalder Temp (65 °C)	0.334	Sheep On Farm (Y)	0.551
Rinse Water Chlor (OA)	-0.874	Scalder Temp (82 °C)	-0.001	Water Source (Well)	0.160
Day of Year	-0.002	Rinse Water Chlor (N)	0.162	Length Feed Restrix Process	0.042
		Rinse Water Chlor (OA)	-0.029	Scalder Temp (65 °C)	0.054
		Transport Time (3)	-0.603	Scalder Temp (71 °C)	0.032

	Day Of Year	-0.002	Rinse Water Chlor (N)	0.746
	Flock Age Days	0.015	Day of Year	-0.0001
	Breed (FR)	-0.015	Flock Age Days	-0.005
	Breed (RR)	-0.301	Breed (FR)	-0.052
			Breed (RR)	0.013