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Quantitative Microbial Risk Assessment for Parts, Ground, and MSC Poultry Product Including Intervention Analysis and Exploration of *Enterobacteriaceae* as an Indicator Organism in Poultry Processing

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Poultry Science

by

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Abstract

Samples collected at five different large bird poultry processing facilities over a period of 7 months from prescald to post debone locations were enumerated for *Enterobacteriaceae*, *Salmonella* spp., and *Campylobacter* spp. and the results were used to create Quantitative Microbial Risk Analyses (QMRA) models for parts, ground, and mechanically separated chicken (MSC) products. Sensitivity analyses indicated the points in the process at which reductions would be most advantageous to the endpoint and simulation models were run to test reductions required to meet the current USDA performance standards.

These data were analyzed to determine the reductions from one node (location) to the next and including outside variables (line speed, presence of a post-pick cabinet, pH, and chemical concentration) in the process that may affect the efficacy of these applications in the process. Stepwise regression analyses were used to determine if there was a relationship between the reductions and these variables. If the relationship was significant, then it was further explored with linear estimation to find the most beneficial point at which each of these factors influenced the largest reduction in either *Salmonella* spp. or *Campylobacter* spp.

Data on poultry products during processing was analyzed to identify for *Enterobacteriaceae, Salmonella* spp., and *Campylobacter* spp. for each sample. These samples were then compared to determine if there existed a statistically significant relationship between *Enterobacteriaceae* and *Campylobacter* spp. and/or *Salmonella* spp. at first processing and for several post-debone products (parts, ground, frames, and MSC).

Results from these analyses indicated that the parts product should be able to meet USDA FSIS Agency standards, but that ground and MSC product would require reductions (starting from pre-scald) that may not be reasonably likely to occur at a processing facility. The use of the

intervention analysis should aid in determining the best intersect of pH and chemical concentration in removing these pathogens from the first process whole bird carcass rinse (WBCR) samples. Analysis of *Enterobacteriaceae* as an indicator organism resulted in a justification for the use of *Enterobacteriaceae* as a predictor organism for first processing WBCR samples.

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Table of Contents

List of Tables

List of Figures

List of Figures (cont.)

Chapter 1 Introduction

1.1 Poultry Production and Foodborne Pathogens

Interest in foodborne pathogens has been strongly focused upon the meat industry (Fegan and Jenson, 2018). The poultry production industry has specifically been associated with increased risk of *Salmonella* and *Campylobacter* infection (Crotta et al., 2017). Recent Agency standards (USDA FSIS, 2016) for these pathogens have been created in an attempt to mitigate the risks associated with consumption of poultry products, claiming to help reduce approximately 50,000 illnesses annually (Consumer Federation of America, 2016). As *Salmonella* and *Campylobacter* are two of the most documented foodborne pathogens in the United States, the focus on these pathogens in poultry production is justified (CDC, 2018).

Salmonella and *Campylobacter* load in poultry products may be the result of either initial contamination at grow-out or cross-contamination during processing. Birds come to the processing facility from broiler farms after being crated on a truck for the transport to the facility. Any pathogens that are on the birds at grow-out will continue on to the facility, as well as any cross-contamination that may occur on the truck (Skarp et al., 2016).

As birds are brought into the processing facility they are off-loaded onto a platform that merges into a belt where there may be contact between contaminated and uncontaminated animals. As the belt runs into the live-hang area, birds are shackled, and after harvesting are moved into the scalder, where hot water flows at a countercurrent to clean physical contamination off the bird carcasses. The scalder and the consecutive picker location are both places where contamination can be spread from one bird to another (Seliwiorstow et al., 2016).

The bird carcasses remain shackled while they go through evisceration and more interventions, such as inside-outside bird washers (IOBW) and the on-line reprocessing cabinet

(OLR). Once the OLR treatment is complete the birds are un-shackled and immersed in the chiller apparatus, after which they may go through deboning and further processing. The chiller exit is the end of first processing at the facility. Carcasses then go on to debone, where they may be cut into parts, and some of these parts may go to ground product, while frames from the carcasses will go into mechanically separated chicken (MSC) product.

Bird rinses or product samples may be taken throughout this process to monitor pathogen levels at each stage of the process. Bio mapping studies show that counts may be expected to decrease from the pre-scald location to post-chill, and rarely increase at any points thereafter. Although cross-contamination can occur in a process, the flow of the process is rapid and it is unlikely that bacterial load would have time to increase to a meaningful degree. Excluding specific processing situations, the ambient temperature after first processing is such that any research can justifiably consider the predominance of pathogen load recovered as the result of initial contamination of the carcass.

The ground and MSC processes are different from others in that product from multiple carcasses is homogenized and, at these locations, mean $\log_{10}$ *Salmonella* and *Campylobacter* count have been known to be higher than the load found on the product prior to processing in the grinder or beehive. Determining how to reduce bacterial counts for parts post-debone and in ground and MSC product has become a problem that researchers in the poultry industry have sought to solve.

1.2 Quantitative Microbial Risk Analyses

The first quantitative microbial risk analyses (QMRA) were completed to follow the presence of *Salmonella enteritidis* in eggs (Whiting and Buchanan, 1997) and to follow *E.coli*

0157:H7 through a ground beef process (Cassin et al., 1998). These models linked distributions that represented separate events in a process and then used an iterative sampling technique to create a final distribution from which predicted exposure could be determined. Most QMRA exercises follow this format, with many adding an analysis at the end of the exposure endpoints to attempt to forecast the risk of infection through a dose-response, although the power of these projections has been questioned (Fegan and Jenson, 2018).

There have been many QMRA analyses completed for *Salmonella* spp. in poultry and most likely more for *Campylobacter* spp. as a result of the *Campylobacter* Risk Management and Assessment (CARMA) project in the Netherlands (Havelaar, 2004), the results of which helped to further define the nodular format often used in current QMRA designs. In this stochastic approach, locations, or nodes, where counts could increase or decrease due to several factors (intervention, cutting, mixing, or cross-contamination), are investigated with distributions which are linked with a modular design (Nauta and Schaffner, 2008).

The most difficult aspect of creating a QMRA that is representative of a process is the integrity of the data used for its construction (Pradhan, 2001). Most QMRA models are, by necessity, built with data from multiple prior studies or samples from different flocks or facilities. A QMRA built from samples collected as they run through the process would be of benefit to better understand foodborne pathogens in poultry processing.

1.3 Analyzing Pathogen Reduction by Intervention Type and Chemical Concentration

A large section of the literature on the efficacy of specific poultry processing interventions in reducing microbial load is focused on a specific chemical applied at a specific location. Samples are taken before and after application and then the bactericide's success is determined by the

amount of reduction exhibited after application. Many of these studies are completed in a laboratory setting or a pilot plant, where conditions are controlled and media may be inoculated. These are beneficial to understanding the mechanism of an intervention on products but cannot alone indicate if the products being tested will work effectively in a processing environment.

However, sampling to test an intervention at processing is not without its own set of difficulties. Recovery at some locations, specifically post-chill, is considered suspect by some researchers in that the landscape of the bird carcass can hold onto cells even at rinsing (Lillard, 1988; Williams et. al, 2010). Currently there is concern that the use of non-neutralized buffered peptone water in bird rinses may also allow interventions to continue to destroy cells up to recovery. With refined processes, both *Campylobacter* and *Salmonella* have been reduced in the processing environment to an extent that they are difficult to find.

Analysis of these datasets presents even more difficulty when a large frequency of 0's, or indeterminable results, are left to the researcher to define. Because $\log_{10}$ transformation of 0 results are undefined, it is left to the researcher to transform these values to a meaningful result. When sampling is costly and datasets are small, there is some difficulty in teasing out meaningful answers regarding the efficacy of interventions at processing.

1.4 *Enterobacteriaceae* as an indicator organism for *Salmonella* and *Campylobacter*

The use of indicator organisms has proven useful in detecting and defining the behavior of foodborne pathogens in poultry processing (Roccato et al., 2018; Schaffner and Smith, 2004). Indicator organisms act as a gauge from which the increase or decrease of a pathogen, if it were present and had been recovered, it could be predicted. Such information allows researchers to determine if an intervention or process would be successful in inactivating these pathogens without

the cost of extensive laboratory experiments while taking advantage of the variable nature of the processing environment.

Enterobacteriaceae has long been considered an indicator organism (USDA, 2012). It is relatively inexpensive to enumerate (in relation to more costly methods, such as MPN methodology for recovering *Salmonella*). *Enterobacteriaceae* is found in greater numbers than either *Campylobacter* or *Salmonella* so reductions can be readily monitored. As *Salmonella* falls within the large *Enterobacteriaceae* family it is reasonable to consider it as a reliable indicator organism for this pathogen. The goal is to find a relationship, if one exists *between Enterobacteriaceae* and *Campylobacter*, or *Enterobacteriaceae* and *Salmonella*, and to define these terms to an extent that the load these pathogens may be inferred from the indicator organism.

1.5 Elements of the Dissertation

Chapter 2 includes the entirety of the literature review for this dissertation. Included in this section are a discussion of the current industry environment with regard to *Salmonella* and *Campylobacter* and an overview of where these pathogens have been found in poultry procesing. Also covered are the current USDA *Salmonella* and *Campylobacter* performance standards and the progression of these guidelines (FSIS, 2015,2016).

Chapter 2 outlines different types of models that have been used to try and predict the behavior of microbes, from primary models that include only one variable such as temperature, to secondary models that include an additional variable, to QMRA analyses that use multiple distributions to arrive at a predicted frequency of a pathogen in the final product. A discussion of interventions commonly used at poultry processing facilities is included as well as an overview of the use of indicator organisms and their utility in poultry processing follows.

Chapter 3 includes the experimental design and collection details for QMRA models built to represent three product types (parts, ground, and MSC). An analysis of variance was used to separate the data by seasonal cohorts, if necessary, and the uncertainty was included in the models. Sensitivity analyses were used to determine points in the process that most defined the output. Estimated reductions were discussed at points that would allow output performance similar to those defined by Agency guidelines and shown in Table 2.1 (FSIS, 2016).

Chapter 4 is an analysis of select factors present in a poultry production process such as line speed and presence of a post-pick steam cabinet. Included in this study are the pH and concentration of chemical interventions and where the greatest reductions of *Salmonella* spp. and *Campylobacter* spp. occurred within these parameters. Stepwise regression tests were used to define the point at which multiple factors were most beneficial to microbial reduction.

Chapter 5 is a consideration of *Enterobacteriaceae* as an indicator organism for *Salmonella* spp. and *Campylobacter* spp. in a poultry production process. Samples collected at production facilities were prepared for enumeration of all three organisms on the same product. These data were fit to linear models and, if applicable, relationships between the *Enterobacteriaceae* organism and each of the selected pathogens was further defined.

Chapter 6 is a discussion of the results of the previous chapters and how all three studies complement one another. Sources of error are discussed as well as recommendations for research to further define the information provided in this dissertation.

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Chapter 2 Literature Review

2.1 Food Safety Implications of *Salmonella* spp. and *Campylobacter* spp. in Poultry Product

The presence of *Salmonella* and *Campylobacter* bacteria in poultry product is of considerable concern for the poultry industry as consumption of incorrectly prepared poultry that harbors these pathogens has been associated with human illness (Bailey et al., 2001; Bryan and Doyle, 1995; Finstad et al., 2012, Waldroup, 1996). Both *Salmonella* and *Campylobacter* may be found in a variety of environments, but the warm digestive tract of mammals is a particularly welcome host to the microbes.

Salmonella enterica belongs to the *Enterobacteriaceae* family, and has been linked to at least 1.2 million human illnesses a year in the United States (CDC, 2018), with poultry products being associated with approximately 30% of these infections (Foley et al., 2011; McEntire et al., 2014). The incubation period for *Salmonella* illness in humans ranges from 12 to 72 hours post-ingestion and can continue from 4 to 7 days, with physical symptoms of infection including vomiting, abdominal pain, lethargy, and diarrhea (CDC, 2015). The infectious dose for healthy individuals is at approximately 6 $\log_{10}$ (Baird-Parker, 1990), with lower amounts resulting in illness or death among the very young or very old and immune-compromised individuals (Kennedy et al., 2004).

Campylobacter, most specifically, *Campylobacter jejuni*, is also a major source of gastrointestinal illness, and, like *Salmonella*, may be found at a high frequency in raw poultry (Blaser, 1997). *Campylobacter* is currently the third most common cause of gastrointestinal illness behind *Salmonella* and *E. coli 0157:H7* (Mattia et al., 2018), and is often contracted from large-scale preparation environments, such as cafeterias or restaurants (Friedman et al., 2004). *Campylobacter* illnesses often occur sporadically, rather than in outbreaks which makes it

difficult to trace to origin of contamination (Corry and Atabay, 2001), and infection can occur anywhere from 2.9 to 6 $\log_{10}$ MPN/g (Acheson and Allos, 2001).

Campylobacter illness can occur 2 to 5 days after ingestion and may be quite mild, only lasting 24 hours, or exhibit more serious symptoms of gastrointestinal illness which can last up to 10 days (Blaser, Taylor, and Feldman, 1983; Food Safety.gov, 2015). Infection can occur at doses as low as 3 $\log_{10}$ (Teunis et al., 2018), and is often associated with undercooked, or poorly prepared poultry (Tauxe, 1992).

Salmonella and *Campylobacter* have both been found in every point in the poultry production process, with colonization possible from the breeder facilities throughout production. Vertical transmission of *Salmonella* has been documented at breeder locations, with the bacteria transferred to eggs from colonized laying hens (Berchieri et al., 2001), and studies have documented cross-colonization from eggs at the hatchery (Bailey, Cox, and Berrang, 1994). Some studies suggest that *Campylobacter* does not appear to transmit easily from breeder to progeny (Callicott et al., 2006; Shanker et al., 1985), but others indicate that the bacteria can be transmitted from hens to broiler flocks (Cox et al., 2002).

The same *Salmonella* serovars found in feed mills have been shown to exist in broiler houses and at processing facilities (Corry et al., 2002), whereas the presence of *Campylobacter* doesn't appear until 2 to 3 weeks after chick placement, even though it is assumed that birds have been exposed to the organism (Newell and Fearnley, 2003). Transfer of both pathogens to the processing facility is evident, with the production facility utilizing a variety of interventions to reduce loadings of the organism on broiler carcasses prechill.

Second processing steps at the broiler facilities are focused on lowering the incidence of raw product cross-contamination as it progresses through value-added steps such as deboning, cut-up, grinding, or mechanical separation. Each successive production step increases exposure to belts, storage bins and handling, along with increased time at temperatures that may be conducive to microbial growth.

Traditionally, process analyses have accepted that levels of *Salmonella* and *Campylobacter* are low postchill, and that any increase in mean counts throughout the process are indicative of cross-contamination. However, recent studies have questioned the accuracy of post-chill rinse results and whether low counts are simply the result of residual kill from chiller intervention chemicals rather than a reduction in carcass load. There is also evidence that bacterial load on carcasses post-chill may be under represented due to the way bacteria can lodge in skin and muscle surface (Lillard, 1989).

Poultry consumption has increased significantly in the United States since the 1960's due to increased availability of both raw and value-added products and its lower price-per-pound in relation to other protein sources (Goodwin, 2005; Thurman, 1987; Windhorst, 2006). Along with increased production is consumer emphasis on food safety which, in turn, requires better methods of defining food processing methods.

2.2 USDA Pathogen Standards for *Campylobacter* and *Salmonella* in Poultry Processing

In 2012, in response to an outbreak of *Salmonella* Heidelberg in ground, NRTE turkey product, the USDA-FSIS issued new pathogen performance standards, incorporating parts, ground, and comminuted poultry into its *Salmonella* Verification Sampling Program for Raw Meat and Poultry product, and requiring all facilities producing comminuted NRTE product to

reassess their HAACP plans (FSIS, 2012; FSIS, 2013). A second specification was the commencement of the NRTE Comminuted Poultry Sampling Program, which increased the established 25 gram sampling parameter to 325 grams (FSIS, 2013).

At the same time, USDA began the collection of a baseline dataset (from January to August, 2012 for parts, and from June 2013 to January 2014 for comminuted product), the results of which were used in determining performance parameters for both *Salmonella* and *Campylobacter* presence in poultry product which was published in January, 2015 (FSIS 2013; FSIS, 2014; FSIS, 2015a). Performance standards were based on the goal of a 30% reduction for *Salmonella* and a 32% reduction for *Campylobacter* resultant foodborne illnesses by 2017. The result was further reduction of the acceptable rate of positives for the poultry industry (FSIS, 2015a). (Table 2.1).

Table 2.1. USDA *Salmonella* and *Campylobacter* Performance Standards

Product Type	***Salmonella* prevalence**	***Campylobacter* prevalence**
Chicken Parts (4 lb.)	15.4%	7.7%
Ground and MSC	25%	1.9%

The established discrete (presence/absence) *Salmonella* and *Campylobacter* performance standards in raw poultry products have limitations: First, collecting data from products at various facilities, and then making decisions on the average of positive samples found is predicated on the idea that there is a specific tolerance level of *Salmonella* and *Campylobacter* positives in a sample set, above which the risk of public exposure exceeds the gain of the food entering the

market. Second, the inherent assumption in this sampling strategy is that these pathogens are going to be homogenously distributed throughout each sample, and that every constituent gram within a sample will exhibit the same enumerative pattern (Corry et al., 2007). In fact, evidence suggests that if one were to separate a 325 gram portion into 50, 1-gram samples, only a portion of those 1 gram samples would contain enough *Salmonella* to result in a positive test (Zelenka, 2014). Also of note is that the limit of detection (LOD) used to determine *Campylobacter* frequency positives in the baseline data for ground/comminuted chicken product was 6 cfu/g (FSIS, 2015), which increased the probable frequency of non-detects in the distribution. This number may only be lowered by lowering the LOD (Busschaert et al., 2010).

Reducing the frequency of *Salmonella* or *Campylobacter* positives does not necessarily mean that the level of pathogen on the product has been decreased. Conversely, a high percentage of positive results does not automatically indicate an increase in count. Studies with regard to *Salmonella* enumeration on raw poultry show that variation of the counts in a sample are made up of a preponderance of low counts (Mead et al., 2010; Waldroup,1996), and a very small frequency of higher end results (>1 cfu/g) which may be reduced through intervention or thermal inactivation. Thus, further intervention may decrease the load in the process, but may not necessarily decrease the frequency of positives. One would have to reduce the load to less than the LOD before a significant reduction in the number of positives would be exhibited.

In addition, the implementation of performance standards for *Campylobacter* spp. and *Salmonella* spp. follows the assumptions that all strains (14 for *Campylobacter*, and > 2000 for *Salmonella*) have the same likelihood of occurrence and pathogenicity. In reality only a portion of the serotypes for these pathogens will cause illness, with some more likely to result in illness than others (Lianou, 2017; Silva et al., 2011)

Measures of prevalence in comminuted poultry are also not sufficient to guide investigators through reductions in the process (Brichta-Harhay et al., 2007). Studies focused on reduction of counts, rather than elimination of positives, by nature tend to be more informative, as it is not clear what range of counts is encompassed within a "positive" sample. Enumeration allows for examination of reductions through the process, as it is questionable as to whether pathogen load will ever be 100% eliminated (Mead et al., 2010). Additionally, a reduction in frequency does not necessarily equate to a reduction in risk (Duarte et al., 2016; Pouillot et al., 2015).

There has been significant support that agency guidelines for poultry product should be based upon *Salmonella* enumeration, most specifically count reduction, as opposed to presence/absence measures (McEntire et al., 2014; Singer, 2014). However, obtaining enumeration data is costly and time consuming. Investigations of both ground poultry and parts for *Campylobacter* also suggest that it is the drop in numbers of a pathogen, as opposed to the percent positive, that will reduce risk (Nauta and Schaffner, 2008).

2.3 Risk/Reduction Analyses in Poultry Processing

Traditional analyses for microbial load on poultry detect the presence or verify the efficacy of process interventions by comparing rates between locations or treatment types (Parveen et al., 2007; Whyte et al., 2002). For studies where *Salmonella* or *Campylobacter* are enumerated, the mean counts between locations, or between control and treated samples are compared (Corry and Atabay, 2001; Jorgensen et al., 2002). However, in the past twenty years, the use of inferential statistics to predict the risk of pathogen load on poultry product has become increasingly common, and the methods utilized in these investigations has become well-defined in the industry as Quantitative Microbial Risk Assessments (QMRA) (Buchanan and Whiting,

1996; Whiting, 1995). These models allow researchers to predict either increase (through cross-contamination or harborage), or reduction of microbes in a product or process. Fitting these models to appropriate distributions can allow presumptions to be made about populations from which samples were selected.

Analysis of distributions or product samples, rather than point estimates (Duarte et al., 2016), allow for scenarios that include the variability found at a processing facility and the uncertainty of both sampling and plating methodologies. Models using enumerative data are more illustrative of the impact of reductions in a process as continuous distributions highlight the cumulative probability of specific loads of pathogens as opposed to presence or absence at a specific LOD.

2.3.1 Predictive Microbiology

Initial microbial models were used to follow the growth or inactivation of organisms under controlled conditions (Fakruddin et al., 2011; Lopez et al., 2004). Empirical functions derived from these early analyses allowed the researcher some level of predictive confidence to the behavior of microorganisms under a fixed temperature through time. Primary growth models utilized a number of forms (linear, logistic, and sigmoidal-shaped distributions), and were capable of mathematically describing the stationary, lag, and growth phases of microbes (Baranyi et al., 1993; Vijay et al., 2006). The basic equation (Equation 1) for exponential microbial growth (or inactivation, if multiplied by -1) describes an increase through time as influenced by temperature:

$$\frac{dN}{dt} = \mu N, \tag{2.1}$$

Where, N indicates the number of micro-organisms ($\log_{10}$ transformed cfu/ml), t represents elapsed time, and μ is the (constant) rate of growth through time (Baranyi and Roberts, 1995).

However, bacteria go through the lag, exponential, and decreasing phases throughout its life; this has been described by 3-phase functions, such as the modified Gompertz in figure 2.1 (Zwietering et al., 1990).

Conversely, inactivation (thermal processing) models have been used to predict the rate of decline of a microbe. These models may be used to determine D-values (decimal reduction), and are an indicator of the time it takes for an organism to be reduced at a specific temperature by 1-log, or 90%. Linear models provide a good fit as the decrease in viable organisms is somewhat consistent at each time cohort (Whiting, 1995). Thermal inactivation models are the reverse of the growth models.

Secondary, or dynamic, models took factors from the primary models and added other variables, such as pH, A_w, or fluctuating temperatures in order to determine their effect on microbial growth or inactivation. In these models, the level of contamination was the target variable which was influenced by the levels of each of the other factors. Ratkowsky et al. (1983) or Gompertz (Winsor, 1932) equations were commonly used to create these secondary models. Both growth and death curves were created to predict microorganism behavior in a variety of substrates until the 1990's, when questions arose regarding to their applicability to processing environments (Dennis et al., 2002). As the growth and inactivation models were almost entirely constructed from data based on the behavior of pathogens in broth, their benefit was questioned as there was a higher temperature resistance (higher D-value) of certain microbes, such as *Salmonella* or *Listeria innocua*, in chicken product in a plant as opposed to those in broth (Murphy et al., 1999; 2000). Although some researchers felt that if all other variables were similar the influence of broth as opposed to a factory environment was negligible (Fakruddin et al., 2011), it was suggested that models built in a laboratory environment were devoid of

Table 2.2 Examples of Growth and Inactivation Models

Function	Type	Source
$\sqrt{r} = b(T - T_{min})\{1 - \exp[c(T - T_{max})]\}$	Growth	Ratkowsky (Ratkowsky et al.,1983)[1]
$g(t) = a + ce^{-e^{b(t-m)}}$	Growth	Gompertz (Baranyi and Roberts, 1995)[2]
$y = Aexp\{-\exp\left[\frac{\mu_m * e}{A}(\lambda - t) + 1\right]\}$	Growth (3-phase)	Modified Gompertz (Zwietering et al., 1990)[3]
$log_{10}(N) = log_{10}(NO) - \frac{k_{max} * t}{\ln(10)}$	Inactivation	Log-linear (Scanlon et al., 2013)[4]
$log_{10} S(t) = -b(T)t^{n(T)}$	Inactivation	Weibull (Corradini and Peleg, 2009)[5]

[1] where $\sqrt{r}$ is the constant rate of growth, b is the regression coefficient of rate of growth at x_i degrees K, and c is a constant by temperature.
[2] where, g(t) is the $\log_{10}$ count, x_{max} is the maximum value of x at time (t), c=Gompertz function constant, and x(t) is value of x at time (t).
[3] where μ_m is the maximum growth rate, λ is the lag time, and A is the maximum value reached.
[4] where k_{max} is the maximum inactivation rate.
[5] where S(t) is the survival rate at time t, and b(T) and n(T) are shape parameters.

competitive exclusion seen in the wild, resulting in a false inflation of the microbe count (Oscar, 2006).

Both growth and death curves were created to predict microorganism behavior in a variety of substrates until the 1990's, when questions arose regarding to their applicability to processing environments (Dennis et al., 2002). As the growth and inactivation models were almost entirely constructed from data based on the behavior of pathogens in broth, their benefit was questioned as there was a higher temperature resistance (higher D-value) of certain microbes, such as *Salmonella* or *Listeria innocua*, in chicken product in a plant as opposed to those in broth (Murphy et al., 1999; 2000). Although some researchers felt that if all other variables were similar the influence of broth as opposed to a factory environment was negligible (Fakruddin et al., 2011), it was suggested that models built in a laboratory environment were

devoid of competitive exclusion seen in the wild, resulting in a false inflation of the microbe count (Oscar, 2006).

These formulas were the result of fitting a curve to the data, and they have been described more as functions than models (Baranyi and Roberts, 1995), as they were only applicable to the environment of a specific experimental design and made certain mathematical presumptions about a process (for instance, as time increases, growth or decline will occur at a constant rate). Creating a predictive model of bacterial load required that these data be collected and analyzed as a mechanistic process. Attempts to apply predictive knowledge to food processing systems, and to predict risk based on process data resulted in the creation of systems to define food production processes in a mechanistic structure.

Researchers began to combine these primary and secondary type modeling exercises and added data that were directly from the process (mechanistic) as opposed to entirely from the laboratory and theoretical analyses (empirical) to attempt to answer questions about food production processes.

2.4 Quantitative Microbial Risk Analysis (QMRA)

Quantitative Microbial Risk Analysis (QMRA) models combine the structural concept of growth and inactivation models with process data to predict risk at the endpoint of production. The first QMRA analyses for poultry were conducted to forecast the threat of *Salmonella enteritidis* in eggs (Whiting and Buchanan, 1997). These earliest exercises focused on combining earlier empirical growth and inactivation models with statistical simulations in an attempt to control for the variability found in microbe distributions (Lammerding and Fazil, 2000; Oscar, 1998).

Although there is some difference in their order of appearance in contemporary research, there is general consensus with regard to the categories included in a risk analysis (Zwietering and Van Gerwen, 2000). The accepted schematic is represented in Figure 2.2. For most QMRA analyses, hazard identification has already been completed at the point of initial design. Hazard characterization aids in defining the critical process points for microbial load, and exposure assessment is defined by the output of the distributions. Dose-response may be used at the output to determine relative risk at consumption. An exposure assessment may be defined in lieu of a dose-response analysis when the risk levels have yet to be defined, or when the researcher is hesitant to attach a risk to a specific microbial load. In these cases, the researcher illuminates the risk and weight of exposure and the target is focused on reduction of the same (Zwietering and Nauta, 2007).

Traditional methods for QMRA analysis include the fitting of distributions for each variable, or link in the process, in which the microbial load has an opportunity to change. These nodes are then linked, and the data therein randomly chosen from each distribution through an iterative simulation process. The form is as follows:

$$\int (x_e) = \int \left(x_{i_1}, x_{\ldots\ldots\ldots n_1}\right) \pm \int (x_{i_2}, x_{\ldots\ldots\ldots n_2}) \pm \int (x_{i_n}, x_{\ldots\ldots\ldots n_n}) + \varepsilon_{123\ldots n} \qquad (2.2)$$

where, $\int (x_e)$ is the final distribution at process end. The final outcome is the result of the iterative selections from each of the nodes in the process ($x_{i1}\ldots x_{i2}\ldots x_n$) in addition to the error in the modeling process.

The approach seeks to capture probabilities of pathogen occurrence within a system (Nauta, 2002). For instance, Cassin (1998) followed the load of toxin-shedding *E.coli* through a series of steps in a ground hamburger process, and Nauta (2000) fit empirical *Campylobacter* data at specified "modules" throughout a poultry production schemata. Each of these analyses

focused on the change in count distribution through a fluid process, rather than the change in bacterial count in a static environment.

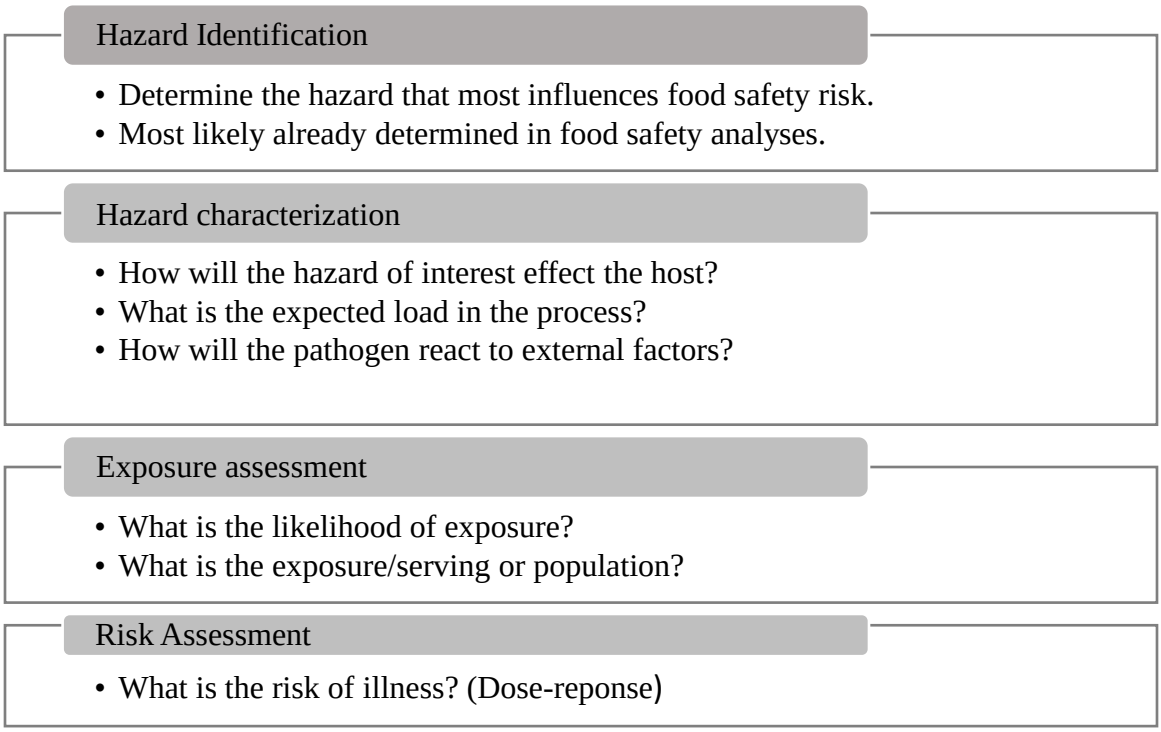

Figure 2.1 Components of a Risk Analysis

These "pathway" (Nauta, 2002) models may then be used to determine risk at process output (see Table 2.2 for examples). Different process mechanisms, such as chemical intervention or mixing, or grinding, that result in changes in both count/frequency of pathogen and distribution of the same may be fit and placed within the framework of the process to allow prediction of output. Further, the researcher may be able to infer the overall impact of increase or production at different stages in the production line.

What process flow that a researcher may choose will depend as much upon available data or sampling opportunity as upon the research question of interest. Some cross-contamination studies focus on the end of production and work to assess consumer risk (Carrasco et al., 2012;

Possas et al., 2017). Others focus on the transfer of microbes within a process (Yang et al., 2002). These analyses use available finished product data, but, due to lack of available within-flock data, must rely on cobbling together records from several analyses to predict risk at handling or consumption.

Many studies are considered "farm to fork" as they follow data from grow-out to the end of production, to consumption. Other efforts begin with the process at the facility, as companies are consigned to deal with the load that comes in the door. Overall, the research question should be the driver for the design, and the data collection should follow this process (Havelaar et al., 2008; Zwietering and Nauta, 2007). For all QMRA research, the focus is on risk (Zwietering and Nauta, 2007), whether that is risk of exposure at the end of a process or risk of consumption at retail.

Early QMRA studies were assessed with available point estimates (mean, standard deviation) from which distributions were chosen based on either assumption or *a priori* knowledge. Later models became more complicated, with multiple distributions being fit for different elements in the research design. Each of these distributions represented a different location where the microbial load could either be increased, reduced, or the distribution changed in some way, such as blending, partitioning, or cross-contamination (Nauta, 2001). Whether they were named Process Risk Models (Cassin, 1998), or Modular Process Risk Models (MPRM) (Nauta, 2002; Nauta and Schaffner, 2008), or even a Dynamic Flow Tree Model (Marks et al., 1998), the object was to partition the points where the pathogen load (or distribution) would be expected to vary from the successive step and model each of these nodes with a different distribution.

The majority of these analyses have used data amassed over multiple studies and a considerable period of time, without the luxury of data collected throughout the same process. Data are usually combined from many different studies, with locations being chosen by available data, not a priori knowledge of the most valuable places to collect (Zwietering and Van Gerwen, 2000). The reason for this is twofold: Most QMRA researchers don't have access to a processing facility, and most processing facility employees are not collecting data to complete a risk analysis. As such, a process model derived from data collected through each specific process flow is of significant value to the food processing industry.

QMRA processing models have traditionally focused on each step in the process in which the bacterial count of interest may reduce, increase, become homogenized, or contaminate other product. These points in the process are helpful in designating process flow, although the flow length analyzed is often influenced by the time and money required to collect samples. Many QMRA studies exist that were created with data collected from several processes, over different time periods. Although these provide utility for food safety research, a similar study, completed with each sampling event consisting of product from the same process, would arguably provide more valuable information with regard to changes within a process.

Process flow is the natural result of the research question, and decisions need to be made based on physical limits for sampling within a facility (Havelaar et al., 2008; Zwietering and Nauta, 2007). If the desirable outcome is to provide information on specific levels of contamination at specific points, then a "farm-to-fork" model may be the best choice. Cross-contamination studies will follow the process, focusing on locations where microbial transfer could occur (debone belt or blending). Industry research often focuses on the most desirable point in the process to reduce pathogen load to achieve a specific endpoint (usually an internal or

Agency guideline). If reductions are not being determined at the farm level, then the most efficacious pathway would run from the beginning of processing (plant entrance) to the end of

Table 2.3 QMRA research on poultry processes

Origin	Product	Microbe	Pathway	Source
United States	Liquid eggs	*Salmonella enteritidis*	"farm to table"	Whiting and Buchanan, 1997
United Stated	Whole Broilers	*Salmonella*	Retail/Transport/Cooking/ Serving/Consumption	Oscar, 1998
Netherlands	Whole Broilers	*Campylobacter*	Cross-contamination	Van der Fels-Klerx et al., 2005
Netherlands	Chicken fillet and table eggs	*Campylobacter* and *Salmonella* spp.	Retail to consumption	Evers and Chardon, 2009
Japan	Whole Broilers	*Campylobacter*	1st processing/Cross-contamination	Hayama et al., 2011
Denmark	Whole Broilers	*Campylobacter* spp.	Small samples at retail	Christensen et al., 2013
Canada	Whole Broilers	*Escherichia coli*	Red water at Chiller	Munther et al., 2016

MCMC = Markov Chain Monte Carlo

production, choosing locations where microbial counts are most likely to increase or decrease (Havelaar et al., 2008).

2.5 QMRA and Poultry Processing

QMRA analyses for poultry processes began in the late 1990's with predictive models being built for egg production (Whiting and Buchanan, 1997). These models were developed

further when a *Campylobacter* Risk Management and Assessment (CARMA) task force was formed to address the issue of *Campylobacter* infection in the European Union (Havelaar, 2004, Nauta et al., 2009). For these models, researchers from multiple disciplines used available data to create both empirical and mechanistic models to predict risk of illness from *Campylobacter* consumption. Many of these studies focused specifically on grow-out and/or first and second processing, cross-contamination, preparation and consumption, the results of which were used to determine guidelines of logistic slaughter practices to reduce microbial load at the end of the process.

Later, modular flows were created to both identify locations of high pathogen risk and to predict distributions of *Listeria monocytogenes*, *Campylobacter* spp., and *Salmonella* spp. on poultry products after differing intervention strategies. Many of these analyses used either empirical data, or data from several different studies for one modeling pathway. Some of these models included attempts to add a cross-contamination element during processing steps, although the functions used to describe such events would be based on a priori knowledge of cross-contamination in a process.

The use of QMRA in poultry processing is not novel. Multiple studies have been conducted to address levels of both *Campylobacter* and *Salmonella* in poultry products. The desire to develop an applicable farm-to-fork model for poultry processing has been soundly voiced. However, the complexity of following such a large amount of data through the process is both cost-prohibitive, and problematic, given that theoretical statisticians don't often get to sample in chicken plants, and poultry processing managers don't often build QMRA models.

In order to continuously meet new pathogen standards while maintaining quality control, it becomes necessary to develop a mechanistic processing model that is comprised of samples

from one flock (or on a flock by flock basis) that are followed through the actual plant process. Studies that use a patchwork of sampling activities are certainly of use in making inferences about the behavior of microorganisms during poultry processing. A continuous model that follows multiple birds through the process which encompasses data from multiple flocks, facilities, days, and seasons may prove a valuable addition to a larger food safety arsenal.

2.5.1 Process Flow in Poultry Production Facilities and Food Safety

Poultry plants produce product from birds transported from grow-out facilities, meaning that birds enter the production process with (more or less, depending on transport conditions) the same level of contamination at which they left the farm. Once the birds are received at the plant they are harvested and sent to the scalder for cleaning.

Birds that enter the scalder water after electric stimulation indubitably introduce bacteria into the warm water in the troughs. The scalder is a suggested physical vector for cross-contamination in at the processing facility (Russell, 2012). Scalder water temperature can be used to reduce the incoming load on the birds, but temperatures above 150º F may result in discoloration of the bird and cause fat to dissolve in the water (Barbut, 2016). This, in turn, may buffer *Salmonella* cells from the heat and result in higher d-values required for expected biocidal impact. Temperatures high enough to melt fat may also affect the retail value of the bird as yield is lost in the scalder.

The high temperature of scalder water loosens the feathers which are then detached by the picking mechanism. This could also result in cross-contamination of the bird carcasses (Russell, 2015), although the movement of the rubber picker fingers across the carcass may also serve to dislodge organisms from the product (Sams, 2016). Post-pick dip tanks with a variety of interventions are currently being used in an attempt to lower loads going into evisceration.

Throughout first processing (from the unloading of the birds at the facility to evisceration) there are a variety of interventions that may be employed, and are utilized at the discretion of individual facilities. Inside-outside bird washers (IOBW) are presented as a cabinet, where hot water is sprayed around the carcasses while they run through the line. An on-line reprocessing cabinet (OLR) is a cabinet with an intervention chemical (usually an acid) spray that is implemented before the immersion chiller. At this stage the bird carcass is still intact, but the viscera have been removed.

The birds are dropped into the first stage of the chiller, which serves to remove any material from the outside carcass. Depending on the facility there may be two or three chiller stages; the final stage consists of an intervention (often a chlorine or acid) and water chilled to a temperature of $\leq 40^{o}$F. As the preponderance of carcass rinse results are negative for *Salmonella* or *E. coli* organisms post-chill, USDA sampling has focused primarily on these results as indicators of food safety. It is often assumed that the chiller intervention takes the product to a level of zero and is thus the most important intervention in the processing flow.

Some studies have suggested, however, that the low counts after the chiller are not indicative of the actual load, rather, that it is recovery of the microbes that lowers to zero. Scanning electron micrographs of post-chill carcasses exhibit multiple *Campylobacter* cells lodged in crevices of the skin and muscle, which may later be expressed in product samples during secondary processing (Chantarapanont et al., 2003). Birds that have been repeatedly rinsed (Lillard, 1989), or, cut-up directly after the chiller also show higher *Salmonella* counts than what have been expressed from carcass rinses. It is also thought that residual kill of microbes in the rinse bags due to acid interventions may be masking the actual counts on the

carcasses, leading to the USDA suggestion of a 1-minute drip time and replacement of neutralized buffered peptone water for carcass rinses (USDA, 2015b).

Secondary processing (post-chill to pack-out) interventions consist of dip tanks and spray rinses. Unique to each facility, sprays are implemented on lines, particularly debone and cut-up, and dip tanks are employed for cut-up parts to control cross-contamination that may occur during processing activities. Sprays are often placed over chicken frames as they enter the initial grinder before the mechanically separated (MSC) process, or on whole leg or breast trim before going into the grinder. The efficacy of parts dip tanks in a processing environment has not been thoroughly tested, although recent use has resulted in counts low enough that baseline standard reassessments have been suggested.

2.5.1.2 Interventions

Interventions may be divided into physical and chemical types, and chemical treatments may be further divided into categories of organic acids, chlorine, or phosphate interventions. The efficacy of all interventions are dependent upon concentration, water temperature, pH, contact time, and organic load at application (Buncic and Sofos, 2012). Combinations of interventions, tailored to the specifics of the process and facility are utilized to lower pathogen counts on the final product.

Physical interventions are most likely as much a part of a facility's sanitation procedures as chemical treatments and are the first line of defense against organic detritus left on the line during production. Hot water sprays and steam applications are used on belts to reduce bacteria and may be combined with manual scrubbing to lower the likelihood of biofilm formation and clean crevices in equipment.

Other physical interventions include the use of temperature to lower microbe counts on carcasses. Hot water used in scalder and picker locations in first processing are used to lower fecal bacteria and can also kill heat-susceptible *Salmonella* cells. Hot water bird washers may be used to lower counts in primary production after picking and before the chiller. The <4 °C chiller water is useful outside of the chemicals used the in process to kill pathogens at lower temperatures.

Organic acids have pH reliant chemistries and have shown promise in reducing bacterial populations. Acetic, citric, lactic, propionic, and malic acids have been used to reduce *Salmonella* in poultry processing, and become more effective at lower pH levels (Mani-Lopez et al., 2012). These treatments are effective and considered safe for use for both *Salmonella and Campylobacter* reduction (Zweifel and Stephan, 2012). Peracetic acid (PAA), a mixture of acetic acid and hydrogen peroxide, is commonly used in poultry processing in sprays, dips and immersion interventions to knock down microbe counts.

Chlorine-based treatments have demonstrated efficacy in reduction of both *Salmonella* and *Campylobacter* in immersion chillers and sprays in first processing. Chlorine interventions are inexpensive (relative to other chemicals) but their effectiveness may be quickly spent in the presence of high organic loads (Buncic and Sofos, 2012). Studies have shown the highest *Salmonella* reduction from chlorine application came from sprays (Loretz et al., 2010). The lower effectiveness in the chiller could be due to the potential for organic build-up during dwell-time, and, as such would require continuous level checks to remain effective (Zweifel and Stephan, 2012).

Tri-sodium phosphate has been used in poultry processing for decades and has resulted in reductions in both *Salmonella* and *Campylobacter* on carcasses (Zweifel and Stephan, 2012).

Phosphate-based interventions have proven useful in reducing *Salmonella* and *Campylobacter* counts with both sprays and immersion. However, the use of phosphates can result in higher pH values of the medium, making sampling difficult, and can leave a slick surface on the product.

2.5.2. Challenges to Poultry Processing QMRA Models

Poultry processing presents a few novel issues for the application of risk analysis. Each poultry production facility is unique, by geographic location and product type being produced, and processing methodologies can differ. Chemical and physical interventions may differ by both facility and process location, with levels of application differing throughout the day (although within specific parameters). Biological load, which can be influenced by bird size, flock size, or season, can cumulatively affect the efficacy of chemicals used to reduce pathogen count in bird washers, reprocessing tanks or immersion chillers.

Laboratory challenges are also of note when attempting a QMRA for a poultry process. First, lab supplies and recovery techniques are expensive and become more cost-prohibitive as sample size increases. (Without the use of a corporate lab, where multiple samples can be plated throughout a number of days, the chance of recovering a representative sampling of a process would not be possible.) Movement of samples from one location to another, multiple persons in charge of plating the samples and error associated with MPN methodology may all result in decreased accuracy in recovered organisms.

2.5.2.1 Modeling a dynamic system

Risk analyses completed on processing (dynamic) systems require a modeling approach that allows for both the random nature of plant flow and the fact that the microbes being sampled are not exactly the same in their response to environmental influence (Brul et al., 2007). Deterministic mathematics, used in earlier primary models to predict death of a microbe at a

specific time and temperature, are not applicable to processes where there is a great deal of variability (Rodriguez et al., 2016). For process models, the design is driven by the distribution at input and subsequent changes in pathogen load that culminate to the final probability of occurrence and load at output. This type of pattern is a Markov Chain process, where the prior location always influences the condition of the subsequent location. However, in order to make predictions of probability with this type of process, the researcher must add an element of variability. Otherwise the results are nothing more than one deterministic model feeding into another. For this process type, a stochastic model, capable of addressing the randomness inherent in the process is required (Nauta, 2002). Sampling from one location to the next in the process and fitting these distributions results in a chain of distributions, each representing a change in the probability of occurrence and the load distribution of the product. Distribution at the end of one location becomes the beginning distribution at the next location.

This randomized sampling can be completed by using multiple draws from each population. A large sample of draws is required; use of computer simulations for the exercise is implemented. Frequently, Monte Carlo simulation has been used to simulate the variability found naturally in a process. The Monte Carlo algorithm uses repetitive and random sampling that is and allows the researcher to make inferences of the probability of occurrence based on the outcome distributions of multiple draws.

Vose (2000) suggests that even though the Monte Carlo method is “unadulterated”, it uses a method based on a uniform distribution draw, so that the intended distribution fit is not necessarily honored in the sampling choices. Thus, Monte Carlo simulations can be useful for introducing model uncertainty. Another selection algorithm, the Latin Hypercube replaces samples after a draw, dividing the distribution into intervals of similar probability (Vose, 2000).

The shape of the chosen distributions is more likely to be maintained using the Latin Hypercube rather than the Monte Carlo simulation method.

2.5.2.2 Sampling Error

One source of error for a QMRA at a poultry facility is error between samples. Multiple carcass or product rinses, taken over a period of days and months, have the potential to result in differing sampling methods among researchers. As sampling technique may increase variably, analyses (such as the Gauge R and R) that measure within sample difference, might be advantageous. It would be beneficial to industry if a QMRA were completed with all samples assuredly collected with the same method.

To date, there is no published QMRA that uses data collected by the same researcher for each sample. This is indubitably due to the fact that this type of research would take a great deal of time to complete. First, the researcher would have to put aside a year for sampling in order to capture seasonality. And, the quick flow of a poultry processing pathway would require the researcher to move quickly to capture the line of each flock as it moved through the process. This would result in only a small amount of samples being rinsed for each process node each day.

Another issue is shipment time of samples after collection. To get the samples back to a laboratory in a timely manner (in which the researcher could be assured that the microbe count was representative of what was collected at the facility), the samples would have to be quickly moved or shipped after collection. Any delay in this process could result in recovery error once the product arrives at the laboratory.

An additional impediment to successful sampling for a QMRA analysis is the focus on biosecurity at food production facilities. Any production plant will be focused on protecting output from outside tampering or contamination. Poultry processing facilities have these issues

as well as the added concerns of outside interests gaining access to plants to film activities in sensitive areas, such as the live-hang area. For the samples to be collected through the process flow, a researcher would need to have access to a high level of security within the production facility.

2.5.2.3 Recovery Error

A key source of error in microbe recovery is recovery methodology and correct counting of colonies, if such are present in the sample. Buffered peptone rinse water (BPW) from poultry carcass rinses (BPW rinsate) was added to product and massaged before plating. Sample plates may then be read for colony count or may be further processed for positive samples (as in the case of MPN analysis). During any of these steps samples may be contaminated or even lost (Duarte and Nauta, 2015).

Some pathogens, such as *Campylobacter* spp., are fragile and, even if present, may be destroyed before recovery can occur. If samples become too warm after collection, resulting counts may not be representative of what was on the bird at the facility. If samples are plated in an environment that is not completely dry, then growth could occur that would not necessarily have resulted during processing. An anaerobic environment at sample preparation may result in the destruction of cells that require oxygen for survival, thus influencing risk analysis outcome for that product (USDA-FSIS, 2016).

Recovery error may also occur at the production facility, which may be the case when cells are destroyed during sampling. It has been postulated that the low counts recovered post-chill are not necessarily the result of counts being knocked back to negligible frequency, but rather, are the result of cells being lodged deeply in the skin and muscle. Lillard (1989) found that multiple carcass rinses post-chill resulted in continuous *Salmonella* spp. positive results,

often after a rinse that did not yield a positive result. Industry studies have shown that carcass rinses post-chill were negative, yet, skin samples taken from the same carcasses were positive for *Salmonella* spp.

Another source of error is residual kill that can result when a chemical intervention continues to work inside a rinse bag along with the sample and rinse water. Some interventions used specifically to reduce counts on product can continue to do their job while sampling is taking place, resulting in a count that is lower than what was actually on the product. Some researchers argue that the intervention would have reduced counts to the same level in the process, so that the residual drop in counts should not be of concern. However, industry studies have shown that a 1 or 2 minute drip-time for product before rinsing resulted in a significantly different mean *Salmonella* count from samples that were not dripped before rinsing (Anonymous, 2014). Agency response to these concerns have resulted in use of neutralized buffered peptone water (NPPW) for the parts sampling program (USDA-FSIS, 2015). Sampling taking this possible source of error into account would need to either utilize NBPW as a rinsate, or maintain a drip-time of 1 to 2 minutes for each product before rinsing.

2.5.2.4 Distribution Fitting

A reliable QMRA model is predicated upon the chosen distributions being representative of the overall population. The degree to which the theoretical distribution is similar to the actual distribution is an important measure of assumptions made from the model. Model "fit" can be measured by a number of equations (Table 2.4), all which rely upon some measure of the overlap between expected and actual ("observed") results. Occam's razor states that the less complex a system the better. The more the model has to be tailored to the sample data (or "over fit"), the

less likely the model will be for the population. Oftentimes choices have to be made between a better "fit" or ease of use.

The Pearson chi-square goodness-of-fit test is used to compare observed and expected results in data that are categorized into bins for continuous data or categories for discrete data. The chi-square comparison is a good choice when the researcher is interested in specific portions of the distributions being aptly represented. The Anderson-Darling test weighs more heavily to the tails of the distribution and is useful when dealing with exposure, as the right tail of the distribution is where the most risk lies. The Akaike (information criteria) test evaluates the fit of distributions in comparison to one another and is useful when multiple distributions are being considered for the model.

2.5.2.5 Dealing with Zeroes

Another element that may add to the error in a modeling analysis can occur when sampling groups contain a high percentage of either non-detects (ND), zeroes, or $<x_i$, (with x being the limit of detection for the assay. These results may either represent a "true" zero, (absence of the organism), a more rare occurrence where a pathogen is present at the LOD but not detected, or, a situation where the pathogen is present, but at a small enough amount so that it is below the limit of assay detection. As microbial risk assessments have evolved, differing methods to work with distributions that have a high frequency of zeros (otherwise known as "censored" or "over dispersed") have been introduced in the body of research (Barron et al., 2014; Busschaert et al., 2010; Gonzales-Barron et al, 2010; Helsel, 2006, 2009; Lorimer and Kiermeier, 2007; Williams et al., 2012).

In many modeling exercises, counts recorded as less-than the limit of detection (LOD) have been handled by imputation, where the unknown values (which are assumed to include both

"true" zeros and those values that are less than the limit of detection) are all replaced with a value chosen by the researcher. Depending on the researcher, the value is often the LOD itself, or 1 log lower than the LOD, or, half the LOD. These methods can be successfully applied when there is a low frequency of values below the LOD, but may result in the creation of an artificial threshold in the dataset and skew the distribution at higher frequencies of 'zeros'. Imputation may also cause artificial inflation of the mean and move the standard deviation closer to the center of the distribution (Busschaert et al., 2010). As such, this method may not be the most advantageous in datasets with a high frequency of zeros.

The "hurdle method" has been used in recent years to account for the frequency of zeros, or non-detects (ND), by fitting a discrete distribution that accounts for the values below the level of ND ("negatives"), then fitting a continuous distribution to the values above the LOD ("positives"). The hurdle method is one of two types of "zero-inflated" distributions, where the 0 occurrences (negatives, or zeros) are handled in one distribution, a probit, and the count data (positives) are handled in another, a logit. The probit function calculates the odds that any sampled value is at, or less than, the LOD, whereas the logit function models the values above the LOD and is truncated (or, left-censored) at this level. As such, only the probability of "true" zeros are included in this model type. This method may be used with a large frequency of zero or ND values, but, the resultant distribution will still exhibit an artificial threshold above the LOD value.

Another type of zero-modified approach (Duarte, 2013; Helsel, 2009) uses both a discrete and continuous distribution, but the threshold for the continuous data is not set at the LOD, allowing for excess zeros outside the frequency distribution. These models are better able to

handle changes in the frequency of samples less than the LOD than the hurdle or imputation methods (Duarte and Nauta, 2015).

The hurdle approach and the various zero-inflated methods result in output that can be difficult to define if there is a large enough division between the "less than" distribution and the continuous one. Also, the resulting distributions from these models will still exhibit an artificial threshold for the less-than LOD occurrences. This becomes problematic if the frequency of negative values decreases throughout the processing flow, as is the case with some ground and MSC chicken product. In this situation, the researcher is left to determine what the increase actually is when a negative becomes a positive, as these situations are separated in the distributions.

There is one method to address, where the frequency below the LOD and the data above are all represented in a single distribution. In this technique, an assumed shape of the population distribution (often lognormal or Poisson) is created using a MLE (maximum likelihood estimation) analysis, where the frequency of non-known, but less than the LOD values is applied, and the most probable mean and standard deviation of the chosen distribution are determined through a series of bootstrap samplings with replacement. Thus, the most-likely point estimates from the distribution of interest are approximated and the resultant distribution is used to determine the values of the ND samples. Several analyses have compared this to the others and concluded these estimations are the most reliable in inferring the true mean and standard deviations of distributions (Helsel, 2009; Hewett and Ganser, 2007; Lorimer and Kiermeier, 2007; Pesonen et al., 2015; Shorten et al., 2006).

The MLE approach does require the researcher to make assumptions with regard to the shape of the distribution, given the values below the LOD are included. Previous studies have

used Poisson-based distributions, such as the Poisson-lognormal or Poisson-gamma (a Poisson distribution where λ and Γ, respectively, are determined by a lognormal distribution), or the lognormal distribution, with the parameters of these distributions determined by a MLE formula that identifies the most likely outcome based on the probability of occurrence below the LOD. This is completed by a series of iterations, each seeking the best scenario and using the constraints of the chosen distribution with a specified frequency below the LOD assumed.

The Poisson-lognormal has exhibited successful inferential strength for higher count distributions, and the Poisson-gamma distribution appears to work well with lower count samples (Duarte, 2013). These mixed distributions allow for more variability than can be achieved by the use of the Poisson distribution alone (Williams and Ebel, 2012). However, the lognormal distribution remains the suggested choice for analysis of microbial populations (Busschaert et al., 2010; Barren et al., 2014; Lorimer and Kiermeier, 2007), although some posit that the lognormal is more valuable in risk prediction for samples with higher microbial counts (Barron et al., 2014).

2.5.2.6 Variability and Uncertainty

Variability is the natural and expected distance between points in a distribution and represents the stochastic nature of a sample population. Any modeling exercise should include the process variability. Uncertainty is the error in estimating the distribution parameters that result from unknown information about the process. Variability represents the random nature of the process, whereas uncertainty represents the error that may be present in estimates of parameters. (Aren and Zio, 2011; Gongouli and Koutsoumanis, 2015; Vose, 2000). Although variability for the most part indicates between sample differences in the process, it may, to an

extent, be reduced by a tighter research design. Conversely, uncertainty can be contracted by a larger sample size or more study (Zwietering and Nauta, 2007; Vasquez et al., 2014).

Many QMRA models have modeled variability and uncertainty together. However, more recent studies have acknowledged the need to define the separate influences of these two components of the analysis, as it allows the researchers to determine the effect of each on the model's pathway (Gongouli and Koutsoumanis, 2015; Nauta, 2002; Pouillot and Delignette-Miller, 2010; Vose, 2000). There are two ways to effectively separate variability and uncertainty. Variability may be put in the model as a formula and then uncertainty can be added in the simulation, or, variability can be simulated and uncertainty added in the model as a series of hyper-parameters based on simulated samples from the original population (Vose, 2000; Vasquez et al, 2014). If the researcher is able to use a formula for the distribution then it is easy to accommodate uncertainty, but most QMRA analyses use simulation software for the variability and uncertainty parameters must then be added to the simulation.

2.5.2.7 Sensitivity Analyses

Sensitivity analyses determine what inputs have the most influence on model outputs by using either regression or Spearman (rank-order) correlation indices to determine the strength certain variables have on the end distribution. In a sensitivity analysis, the effect of changes made to input variables on the outcome distribution are calculated and the variables with the largest influence are identified (Vose, 2000). By stretching the parameters of the input distributions to reasonable limits one can simulate the endpoint, leading to valuable information about the most beneficial location to reduce microbe counts in the process. Forecasting the effect of processing interventions can determine target reductions to reach a desired endpoint.

2.6 Processing Interventions

Birds that enter the scalder water after electric stimulation introduce bacteria into warm water. The scalder is a suggested vector for cross-contamination in at the processing facility (Russell, 2012). Scalder water temperature can be used to reduce the incoming load on the birds, but temperatures above 65º C may result in discoloration of the bird and cause fat to dissolve in the water (Barbut, 2016). This, in turn, may protect *Salmonella* cells from the heat and result in higher d-values required for expected biocidal impact. Temperatures high enough to melt fat may also affect the retail value of the bird through yield loss in the scalder.

The high temperature of the scalder water loosens feathers that are then detached by the picking mechanism. This could also result in cross-contamination of bird carcasses (Russell, 2015), although movement of the rubber picker fingers across the carcass may also serve to dislodge organisms from the product (Sams, 2016). Post-pick dip tanks with a variety of interventions are currently being used in an attempt to lower bacterial loads going into evisceration.

Throughout first processing (from the unloading of birds to evisceration) a variety of interventions may be employed, utilized at the discretion of individual facilities. Inside-outside bird washes (IOBW) are presented as a cabinet in which hot water is sprayed around the carcasses while they run through the line. An on-line reprocessing cabinet (OLR) is a cabinet with an intervention chemical (usually an acid) spray that is implemented before the immersion chiller. At this stage the bird carcass is still intact, but the viscera have been removed.

Birds are dropped into the first stage of the chiller, which serves to remove any material from outside the carcass. Depending on the facility, there may be two or three chiller stages, the final one consists of an intervention (often a chlorine or acid) and water chilled to a temperature

≤ 4 ºC. As the preponderance of carcass rinse results are negative for *Salmonella* or *E. coli* organisms post-chill, USDA sampling has focused primarily on postchill results as food safety indicators. It is often assumed that chiller intervention takes the product to a level of zero and is thus the most important intervention in the processing flow.

Some studies have suggested, however, that low counts after the chiller are not indicative of the actual load, rather, but rather it is microbe recovery that lowers to zero. Scanning electron micrographs of post-chill carcasses exhibit multiple *Salmonella* cells lodged in crevices of the skin and muscle, which are later expressed in product samples during secondary processing. Birds that have been repeatedly rinsed (Lillard, 1989), or cut-up directly after the chiller, also show counts higher than that from carcass rinses. It is also thought that residual kill of microbes in rinse bags due to acid interventions may be masking the actual counts on carcasses, leading to USDA's suggestion of a 1-minute drip time and replacement of neutralized buffered peptone water for carcass rinses (USDA, 2016).

Secondary processing (post-chill to pack-out) interventions consist of dip tanks and spray rinses. Unique to each facility, sprays are implemented on lines, particularly debone and cut-up, and dip tanks are employed for cut-up parts to control cross-contamination that may occur during processing activities. Sprays are often placed over bird frames as they enter the initial grinder before the mechanically separated (MSC) process, or on whole leg or breast trim before going into the grinder. Parts dip tank efficacy in a processing environment has not been thoroughly tested, although recent use has resulted in counts low enough that baseline standard reassessments have been advised.

The most common intervention chemicals used for secondary processing are acid blends, such as peroxyacetic acid (PAA), Acidified Sodium Chlorite, and Hypochlorous acid. Studies

completed in laboratory settings are predominately focused on pre-dip vs. post-dip reductions, whether this is percentage of positive results or a log reduction of enumeration *Campylobacter* or *Salmonella* counts. Again, these reductions have not been followed throughout the mechanistic process flow and may be difficult to define if the load during processing is too low to determine a significant decrease in pathogens. The concentration of intervention (in ppm) is often noted and differing levels have been tested for efficacy. However, as standards become more defined it becomes necessary for industry to find the right combination of concentration and pH for the largest impact of any given intervention chemical.

2.7 Indicator Organisms

The use of indicator organisms is beneficial when the organism of interest is found at very low levels or low frequency during experimental sampling. Often, when attempting to answer questions with regard to level of contamination before and after an intervention step, an inoculation study will be completed. However, when dealing with pathogenic organisms the research must be conducted in a laboratory setting. Although laboratory experiments may be useful tools in assessing the efficacy of different reduction strategies, they are not a viable method to capture the mechanistic process that occurs in processing facilities.

An indicator organism should be found in a readily available quantity in order to be useful. Counts should be high enough that a reduction can be determined if an intervention is applied. Such an organism should also be similar enough to the bacterium of study that they would react the same way to treatments or environment under study or increase in count in a similar fashion (Shaffner and Smith-Simpson, 2014). Of particular benefit to the processing industry would be acceptance of indicator organisms which are more easily recovered and

quantified than pathogens which currently require more extensive methods of count determination.

Index and indicator organisms have been used with varied success. Indicator organisms are often sampled as a yardstick for determining process cleanliness. Index organisms are substituted for an organism that could affect food safety. For instance, Aerobic Plate Count (APC), as well as *Aeromonas* are indicator organisms for process hygiene, or overall cleanliness, in a facility (Saini et al., 2011; Shaffner and Smith-Simpson, 2014). Generic *E. coli* is considered indicative of fecal contaminant (Handley et al., 2015), and thus, may be used as an index organism for pathogenic bacteria.

Enterobacteriaceae (EB) assays include not one, but many organisms that may be a good indicator of an increase or decrease in microbial activity (Kornacki, 2011). EB is most commonly utilized as a hygiene indicator as its assay recovery will include multiple organisms, such as coliforms, fecal coliforms, *E. coli* 0157:H7, and *Salmonella spp.* The *Salmonella spp.* and EB relationship is of interest in this research, and the research design will be structured to test for efficacy of EB as an index organism for *Salmonella*.

EB also has several characteristics that make up a good index organism for *Salmonella*, such as; being present along with *Salmonella*; its habit of occurring in higher numbers than the requisite organism; and enumeration of EB is more convenient than that of *Salmonella* (Bonde, 1966). Earlier studies have indicated a positive correlation between EB and *Salmonella spp.* reduction (Anonymous, 2015). A relationship between EB and *Salmonella* would be a financially beneficial method for the poultry industry to utilize in making inferences about the levels of *Salmonella* on both product and equipment.

2.7.1 Relationship between Enterobacteriaceae and Salmonella concentration

The relationship between *Enterobacteriaceae* and *Salmonella* on raw poultry has been suggested (ILSI Europe), but has not been successfully established to date. *Salmonella* positive results on specific raw pork cuts were associated with higher mean *Enterobacteriaceae* counts, than those for negative samples (Biasino et al., 2017), but a significant positive correlation has been found between EB and *Salmonella* reduction on bird carcass rinses in 1st processing (Anonymous, 2015).

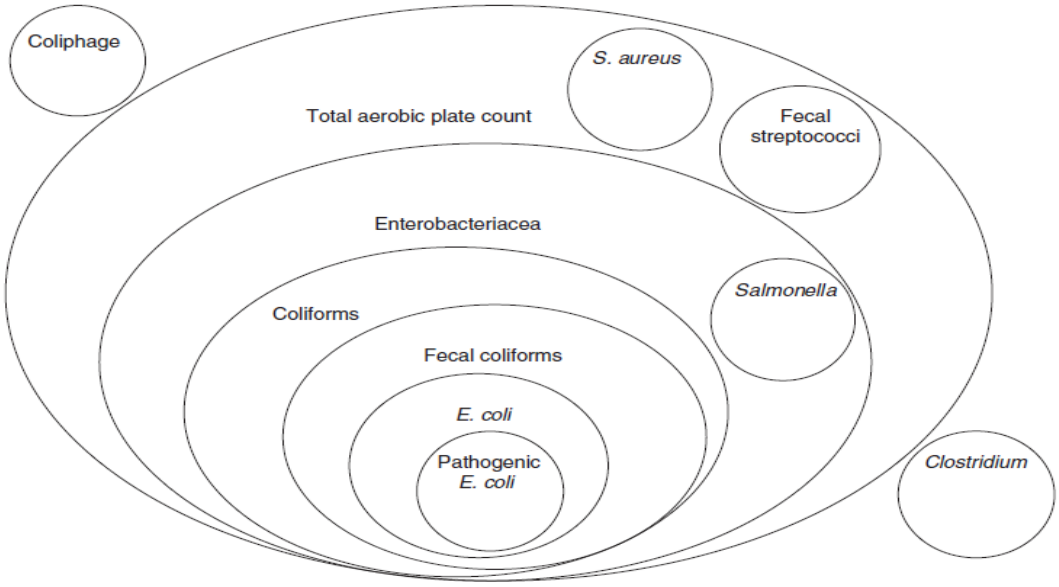

Figure 2.2 Relationship of Microbial Organism
From Schaffner and Smith-Simpson, 2014

As *Salmonella* are part of the *Enterobacteriaceae* microbial family; an increase in one would be expected to result in an increase in the other. However, the strength and shape of that relationship has not yet been defined for carcasses, parts or other poultry products. As *Enterobacteriaceae* is a hygiene indicator organism and *Salmonella* a pathogen, one would need

to act with caution about making any inference based upon the presence or absence of the indicator.

In summary, a quantitative microbial exposure analysis of poultry parts, ground product, and MSC for both *Salmonella spp.* and *Campylobacter spp.* counts is intended to underscore the importance of defining the most beneficial process location. It is expected the results of this study will determine that the locus of greatest reduction in the processes is in first processing locations, and it is proposed that the exposure likelihood can be reduced to a reasonable level if further reductions can be realized in these locations.

Analysis of chemical interventions used in the QMRA sampling distributions should aid the researcher in making assumptions about the expected level of reduction in specific processes. This, in turn, will allow for construction of plausible scenarios where *Salmonella* and *Campylobacter* may be reduced to acceptable Agency food performance standards. If a relationship can be found between *Enterobacteriaceae* and *Salmonella* occurrence and counts, then reductions could be predicted based upon this less costly recovery methodology.

It is expected that results of this study will be of benefit to the poultry industry and will guide further research to help the industry by isolating the most beneficial locations for pathogen reduction and the best intervention concentration and pH level to achieve a targeted reduction for the final product. Results may then be used to find the most cost-effective way to achieve such reductions by removing interventions in locations in which they are not helpful. Results of this study may invite further discourse about the most advantageous method of increasing the efficacy of food safety measures in the poultry industry.

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Chapter 3 Quantitative Microbial Models for *Salmonella* spp. and *Campylobacter* spp. on Parts, Ground, and Mechanically-Separated Poultry Product

3.1 Abstract

Campylobacter spp. and *Salmonella* spp. levels on poultry product are a source of concern for food safety professionals seeking to bring microbial loads to required pathogen standards. Quantitative microbial risk analyses may be used on poultry product processes to predict the output level of pathogens given differing interventions throughout production.

Data from five poultry processing facilities over a series of flocks, during three seasons were collected to determine the expected output load for parts, ground, and mechanically separated chicken (MSC) product. Sensitivity analyses were utilized to determine the most beneficial locations for reduction in the process in order to arrive at pathogen loads that met current agency performance standards (USDA-FSIS, 2015). Simulated reduction scenarios were employed to determine what reduction levels were required to meet these standards.

Due to the large frequency of negative pathogen samples, analyses consisted of multiple lognormal distributions fit to the expected frequency of values below the LOD, utilizing a maximum likelihood estimation (MLE) technique to determine point estimates. Reduction distributions were employed between distributions in a Markov Chain format in order to reduce chaos in the Monte Carlo simulations.

Results indicate that parts standards could be reasonably met with pathogen reductions during the process, whereas much higher reductions, some that may not be reasonable given current intervention limitations, were necessary to bring MSC and ground product microbial levels down to expected parameters. Unexpectedly, results indicated that the frequency of positive samples were higher for the spring/summer and spring/winter seasons for the ground and MSC product than for their summer cohorts.

3.2 Introduction

Microbial modeling in the poultry industry has been used to predict the risk of illness or exposure more frequently since development of computing systems built for sampling large datasets. The results of these analyses have been used to refine processes and predict changes made to lower microbial loads on products. Previous studies have focused upon microbial load at output, the possibility and impact of increase as a result of cross-contamination and risk that may occur from inadequate cooking before consumption (Boysen et al., 2013; Hartnett et al., 2001; Mylius et al., 2007; Nauta and Christensen, 2011; Nauta et al., 2005; Van der Fels-Klerx et al., 2005).

Salmonella is a Gram-negative, rod-shaped pathogenic organism that has been linked to foodborne illness in an estimated 1 million people per year in the US (CDC, 2012). After ingestion of the pathogen, an individual may begin to develop diarrhea, vomiting, or fever within 12 to 72 hours. *Salmonella* has been found in multiple food sources, but is commonly associated with raw poultry products as the organism has been shown to thrive in the digestive tract of the birds.

Campylobacter spp. also thrives in the chicken gut and the hot, humid environment of first processing. Symptoms of *Campylobacter* illness include nausea and vomiting, diarrhea, fever, and possibly bloody stools and can occur between one and seven days of infection.

Both *Salmonella* and *Campylobacter* are of concern in poultry product processing and models that help predict output loads are desired for every product type. Quantitative microbial risk models (QMRA) have been developed for many poultry processes but most have been forced to use data from a mixture of prior studies which have not utilized both flock and facility-

specific data. There are no known microbial risk or exposure analyses for mechanically separated chicken (MSC) product processes.

Microbial risk assessment flows were developed for MSC, ground, and poultry parts (drum and breast) product processes with data collected from multiple flocks and facilities, with each flock being followed throughout the process, from first to the end of second processing. Models were created using the @RISK add-in for Microsoft Excel version 6.3.1. The resultant models were used to create scenarios in which the output may be expected to meet the *Salmonella* and *Campylobacter* performance parameters set by USDA, shown in Table 3.1.

3.3 Materials and Methods

The sampling design followed three product processes (MSC, ground, and parts) from facility entrance to the end of the process line before pack-out. Sampling was completed throughout an 8 month period (December 2015 to July 2016) in order to account for seasonality in the model (Boysen et al., 2013; Williams et al., 2012). Sample season cohorts were spring/fall (temperate weather), summer (hot weather), and winter (cold weather). Each sampling event (day) consisted of 5 samples per location, each of which was analyzed for *Salmonella* spp. and *Campylobacter* spp.

3.3.1 Process Flow

The sampling scheme for the MSC, ground, and parts product began in the live-hang area, directly after bleed-out and before the scalder. This was to capture the microbial load on the birds as they came into the processing facility. Carcasses were then sampled after the picker, followed by samples taken directly after the OLR cabinet, right before the chiller.

Table 3.1 Maximum Allowable Pathogen Standards as Set by USDA FSIS (2015)

Product Type	***Campylobacter* spp.**	***Salmonella* spp.**
MSC/Ground (325 g.)	1.9%	25.0%
Parts (4 lb.)	7.7%	15.4%

Approximately two hours later (after the birds had completed the chiller intervention) samples from the same flock were taken at the end of the chiller. For the MSC flow, frame rinses were taken post debone and then MSC samples were selected randomly from the output chute. For the ground product, whole leg and trim product samples were rinsed, followed by ground product being randomly selected from different sections of the combo. For parts, boneless-skinless breast and drum product was sampled after the dip-tank at the end of the debone line (Figure 3.1). In instances where drum product was not being processed that day, whole leg product was sampled in its place.

Product was followed throughout the process for each sampling exercise to ensure the data was from the same flock (Table 3.1). Each sample collection was randomly selected. Sampling flow by product type are represented in multiple facilities over a period of 8 months to incorporate differing processing methods, seasonality and intra-flock differences. Five samples were taken at each location for every day of sampling.

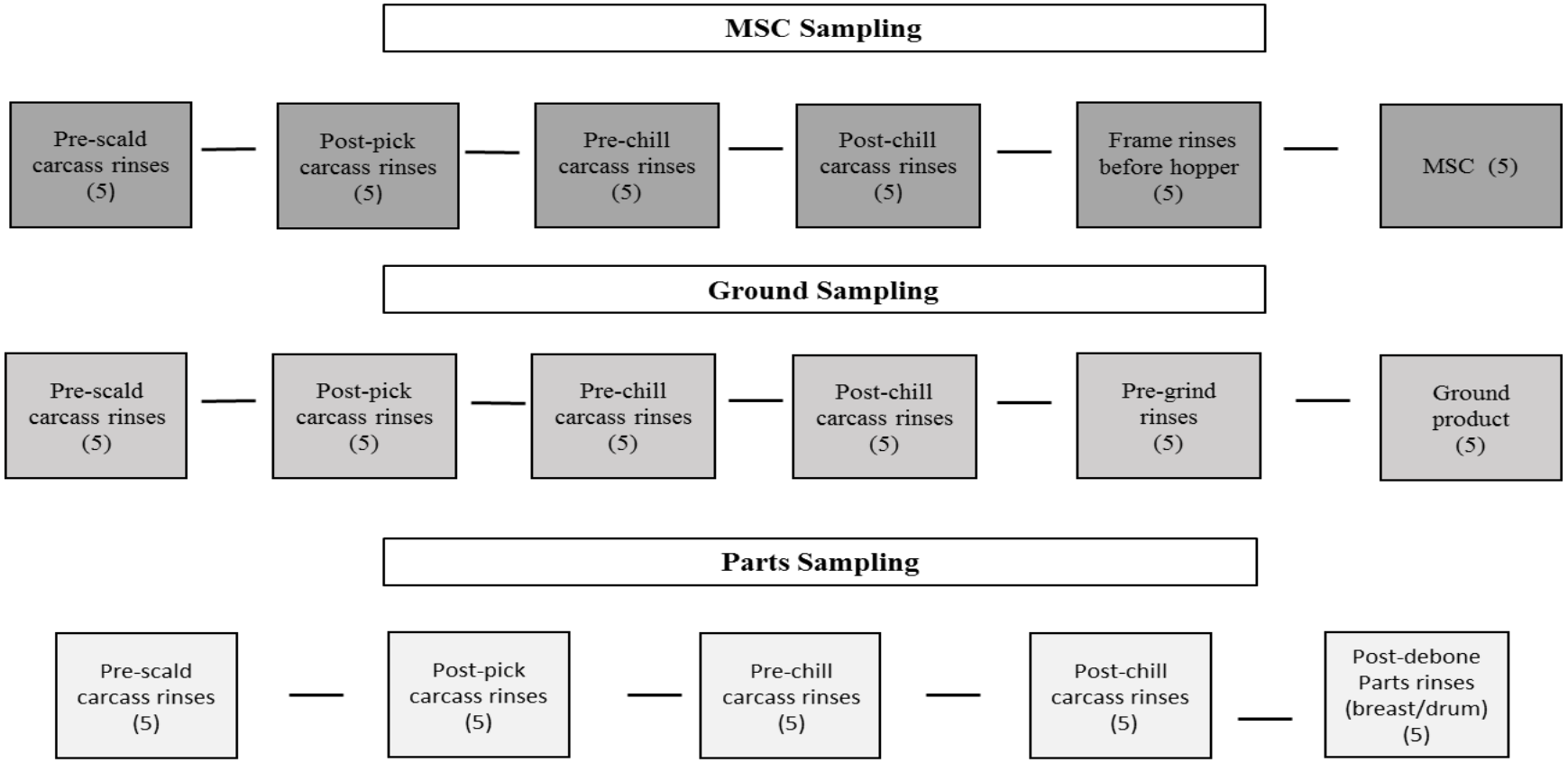


Figure 3.1 Sampling Flow by Product Type
(Locations of each sample taken throughout each process. Number of samples taken in parentheses)

Table 3.2 Number of Samples Collected by Season, Facility, Day and Location
(Number of separate sampling facilities and days by season and overall samples taken)

Product Type	Season	Number of Facilities Represented	Samples per Day	Number of Sampling Days	Number of Samples Recovered (all locations and facilities)
MSC	Fall/Spring Summer Winter	3 2 3	30	5 4 3	145* 120 89*
Ground	Fall/Spring Summer Winter	2 2 2	30	3 2 3	90 60 90
Parts (each type)	Fall/Spring Summer Winter	3 3 3	25	3 5 3	75 120 75

*Not all samples were suitable for analysis

3.3.2 Sampling Methods

For each carcass rinse, a whole bird without giblets (WOG) was collected with an inverted, sterile bag. Carcass samples taken after a chemical intervention were drained, with the carcass drained in an inverted bag for approximately 1-2 minutes each to avoid residual kill of potential microbes during the rinse. Frames and parts product were also drained for approximately 1-2 minutes in order to account for the possibility of intervention holdover on recovery.

Four hundred ml of cooled buffered peptone water (BPW) were poured into the bag, with the liquid saturating both the inside and outside of the carcass. The bag was then twisted to close, and the bag and sample were rocked back and forth for 1 minute. The carcass was then removed from the bag and the remaining solution was poured into the original, sterile sample container, which was then iced and taken immediately to the corporate laboratory for testing.

For the frame rinses, 1 frame each was selected in an inverted sterile bag, with 200 ml of cooled BPW used to rinse the product. Parts were randomly selected out of the combo at 4 lbs. per rinse with 400 ml of cooled BPW used for the rinse.

3.3.3 Recovery Methods

Temperatures for all samples were immediately assessed upon arrival at the corporate laboratory to assure that none of the sample bottle contents had increased above 8ºC, and that none of them had frozen. Resulting data indicated that samples were stored at 2 to 4ºC. Samples were tested for presence/absence of *Salmonella* spp. using polymerase chain reaction (PCR) screening on DuPont Qualicon BAX® methodology. All *Salmonella* spp. positive results were then enumerated using the most probable number (MPN) method. *Campylobacter* was

enumerated via chromogenic agar plates (specific for *Campylobacter jejuni* and *Campylobacter coli).*

Salmonella qualitative analysis was prepared by aseptically transferring 20 ml of rinsate and 30 ml of fresh BPW into a sterile specimen cup for each of the samples. Ground product and MSC samples were weighed at 325 grams each in a filter bag and diluted with 1625 ml of buffered peptone solution and stomached for 30 seconds. All bags were labeled by batch. Controls were prepared and were incubated for determination.

Samples that tested positive for *Salmonella* spp. were then set for MPN. For carcass and parts rinses, samples were directly tubed. For MSC and ground samples, 65g of sample were stomached for 2 minutes in a 585 ml enrichment broth. The 10 ml, 1 ml, and 0.1 ml samples were then set in triplicate tubes. If necessary, .01 ml and .001 ml samples were set in the same manner. Prepared controls and samples were incubated for determination.

Campylobacter rinses were kept on ice until plating with the rinsate representing a 1:1 dilution, whereas ground and MSC product were diluted in 1650 ml of BPW and stomached. For each of 2 plates, 0.50 ml was spread with a sterile instrument across the surface of the agar until the plate appeared to be dry. The inoculum was allowed to dry completely before incubation. Product was incubated at 42 ºC for 36 to 48 hours. At 48 +/- 2 hours the plates were examined for colonies, with the sum of both plates being the reportable count. Presumptive positives were then confirmed.

3.3.4 Modeling Methods

Data were separated for each of the following locations (nodes): prescald, postpick, prechill, directly after the on-line reprocessing (OLR) rinse, postchill, frames (at the end of the debone line), drums and breast product (directly after the parts dip tank), and pre-grind product

(whole leg or trim) directly before the grinder. These nodes were included in the model based upon the designated process flow (parts, ground, or MSC product). Data for each node and product type were separated by season (winter, summer, or fall/spring), and an analysis of variance using (Proc GLM in SAS) was evaluated with season as the main effect. Interaction by facility was not analyzed as one flock per facility visit was collected, resulting in low sample size. Data were $\log_{10}$ transformed for analysis, and negative samples were designated as 0.02 (as the LOD was 0.03) for *Salmonella* spp., and 0.1 (as the LOD was 1 for *Campylobacter* spp.) because the $\log_{10}$ transformation of 0 is undefined. If there was a significant difference between seasons ($p \leq 0.05$), then the data were sequestered in the model, with separate seasons taking different pathways from the node at which they separated.

The raw parts model, consisting of the boneless-skinless breast and drums data may be represented by the following formula:

$$P(f) = C_{I\mu} + (-D_P)_i + (-D_{pc})_j + (-D_c)_k + (-D_{pt})_l + e_{ijkl}, \text{ where:} \quad (3.1)$$

P(f) is the probability of a specific final cumulative distribution at the end of process; C_I is the contamination on the bird carcass before the scalder (incoming load); D_P is the reduction distribution after the picker, $D_{pc,}$ the reduction distribution on the carcass prechill; D_c is the reduction distribution after the chiller; D_{pt} is the reduction distribution of pathogen counts on the parts after the dip tank; and e_{ijklmn} is the random error term.

The ground product and MSC models are similar except that frames were sampled before the MSC grinder and whole leg or trim product was sampled before the grinder. The models for both are as follows:

$$\text{Ground: } P(f) = C_{I\mu} + (-D_P)_i + (-D_{pc})_j + (-D_c)_k + (-D_{pt})_l + (-D_g)_m + e_{ijklm}, \quad (3.2)$$

$$\text{MSC: } P(f) = C_{I\mu} + (-D_P)_i + (-D_{pc})_j + (-D_c)_k + (-D_f)_l + (-D_m)_m + e_{ijklm}, \text{ where:} \quad (3.3)$$

Where, P(f) is the probability of a specific final cumulative distribution at the end of process; C_I is the contamination on the bird carcass before the scalder (incoming load); D_P is the reduction distribution after the picker; D_{pc} is the reduction distribution on the carcass prechill; D_c is the reduction distribution after the chiller; $D_{pt\ is}$ the reduction distribution on the whole leg or trim product (ground model); D_g is the reduction distribution on the ground product (ground model); D_f is the reduction distribution of counts on the frame (MSC model); D_m is the reduction distribution on MSC product (MSC model); and e_{ijklmn} is the random error term.

3.3.5 Distribution Fitting with a High Frequency of Zero Samples

The sampled and recovered data were fit to distributions that best exemplified the spread of data for each location, as well as capturing the frequency of positive values to the whole. There are several ways to handle over dispersed data in quantitative studies. Some modeling exercises have omitted the negative values altogether, which has proven to greatly skew the output. Substituting a value for each of the 0 (or negative) values results in overestimation of the mean and underestimation of the standard deviation (Busschaert et al., 2013; Croghan and Egeghy, 2003; Gonzales-Barron et al., 2010; Helsel, 2006; 2009). Other researchers have chosen the hurdle methodology, a log-probit model where the pathogen frequency of is represented by a discrete function, which is held at 0, with the count data being funneled to a model of best representation (Clark et al., 2008).

Zero-inflated models have also been used to fit distributions with a high frequency of "less than" values. Zero-inflated models, such as the zero-inflated negative binomial, or the zero-inflated poisson-lognormal, use a logit function to model the odds of negative results (or zeros) which flows into a probability distribution of the positive counts. The difference between the hurdle model and its zero-inflated counterpart is that in a zero-inflated distribution the count

model is not held at zero. As a result, "true" zeros are handled in the discrete distribution and other zeros that may occur are handled in the continuous counterpart, which can be more flexible given changes in frequencies in a distribution (Duarte et al., 2014; Duarte and Nauta, 2015). However, a zero-inflated model also requires that the dataset be split into a discrete and continuous format that, at some point, must be modeled together to make inferences about the impact of load reduction at specific points in the process.

For these analyses, a maximum-likelihood estimation (MLE) method was chosen as the best for data fitting. MLE algorithms seek to use a previously defined distribution and to determine the most reasonable parameters based upon the available data. Programs such as Solver in Excel, or Goal Seek in the @Risk software may be used to assess the possible parametric iterations until a reasonable solution is reached. In this case, when more than 15% of the sample distribution consisted of samples less than the level of detection ("negatives") the Solver program in Excel was used to determine the parameters of lognormal distributions.

The MLE was chosen specifically as it allowed the use of a single distribution to approximate the distribution of both the "less than" values and the count values and has proven to be effective in QMRA literature (Busschaert et al., 2010; Hewett and Ganser, 2007). As the Markov Chain format of the simulations requires the nodes to flow into one another, use of a hurdle or zero-inflated approach resulted in bimodal distributions from which simulations of reductions could not be approached. Also, the assumption that any value below the LOD is an absolute "zero" is simply not justifiable and may be influenced by a number of factors such as small concentration of pathogens in product, a too-small sample substrate, splitting samples for testing, or utilization of a test with a high recovery threshold (Duarte et al., 2014; Lorimer and Kiermeier, 2007). In order to model both frequency and count with one distribution, a value was

designated as the LOD threshold, at which any value below that number would also be considered a negative. In the case of *Campylobacter* spp., the LOD was 1 cfu/gram, thus, 0.1 cfu/gram was designated as the highest negative value possible in the distribution. For *Salmonella* spp., 0.03 cfu/gram was the LOD, and 0.02 cfu/gram was designated as the highest possible negative value. The threshold for the frequency of negative samples in a cumulative distribution was then -1.00 log for *Campylobacter* spp., and -1.70 for *Salmonella* spp.

The lognormal distribution was the distribution of choice, as microbial data is well-defined by this treatment (Duarte and Nauta, 2015). Some researchers posit that lognormal distributions are not beneficial to modeling low count data, as the 0 count is undefined in log transformations and that only discrete numbers should be used for modeling bacteria (Jongenberger et al., 2012). However, as bacteria are recovered from products that are parts of a larger sample, and it is the result of ingestion of a larger sample that is of interest, it is not necessarily incorrect to calculate the increase and reduction of the sample as fractions of a whole. And, the use of the lognormal for all over dispersed data sets in the models allows for comparison of the distribution means between locations in the model (Lorimer and Kiermeier, 2007).

The use of two models (one to represent the frequency, and another to represent count values) does not lend itself to simulations where reduced counts are increased throughout the process. When all negative counts are imputed to be one value it is not only unrealistic, but it makes the change from ‘positive’ to ‘negative’ more difficult to model. In the case of this analysis, only the positive samples were enumerated; thus, it makes sense to model with the assumption that many of these negative values are not zero, but are simply counts too low to define (Duarte, 2013). Using MLE to back into a reasonable distribution allows for a more

reasonable output of likely values after simulation. A list of the best fits for the distributions for all models may be seen in Table 3.3.

3.3.6 Handling reduction in the models

The use of Monte Carlo simulations when handling increase or reduction in a chain of distributions can result in a chaotic output if the model does not manage the relationship between successive nodes (Vose, 2000). If a statistically significant relationship between nodes can be established, then a correlation matrix may be used to establish these dependencies. However, the Spearman correlation analysis (PROC CORR in SAS) required imputation of negative or "less-than" values. The high frequency of these values in these datasets resulted in relationships between nodes that may not have been meaningful due to the transformation. As a result, reduction distributions were created to define the relationships between nodes.

A normal distribution of $\log_{10}$ transformed data represented the difference between the mean and standard deviation of neighboring distributions, as$((\mu_{x2} - \mu_{x1}), (\frac{\sigma^2{x_1}}{n} + \frac{\sigma^2{x_2}}{n}))$, where n represented the number of simulations used to define the measurement uncertainty for final distributions (n=100). Using a reduction distribution allowed the chain of events to be connected, without the assumption of a significant correlation between locations, which could not be justified due to the multiple transformations at a censored value. Using reduction distributions also allowed the relationship between locations to be illustrated with introducing results that could be meaningless due to the iterative reductions available in an unrestrained Markov Chain Monte Carlo simulation (Table 3.4).

Table 3.3 Selected Model Distributions

Parts Process/*Campylobacter* spp.					
Prescald Normal(Normal (4.40,0.054),0.98)	Postpick **Summer**: Extreme value (2.39,0.74) **Winter/Spring:** Extreme minimum value (2.45,1.07)	Prechill **Summer:** Extreme minimum value (1.73,0.47) **Winter/Spring:** Normal (-0.58,0.47)	Postchill Normal (2.58,0.77)	Drums/Breast Normal(-2.58,0.77)	
Parts Process/*Salmonella* spp.					
Prescald **Summer**: Normal(Normal (1.13,0.71), 1.24) **Winter/Spring:** (Normal(Normal (0.15,0.80),1.35)	Postpick **Summer:** Normal (-1.43,1.04) **Winter/Spring:** Normal (-1.35,0.90)	Prechill **Summer**: Normal (-1.56,0.80) **Winter/Spring:** Normal (-2.00,0.20)	Postchill Normal (2.22,0.30)	Drums Normal (3.00,0.50)	Breast Normal (2.31,0.61)
MSC Process/*Campylobacter* spp.					
Prescald Normal(Normal (4.22,0.51),1.28	Postpick Triangular(1.86,4.5,4.05)	Prechill **Summer:** Normal (1.64,0.34) **Winter/Spring:** Normal (-0.12,2.00)	Postchill Normal (2.00,0.53)	Frames Normal (1.72,0.53)	MSC **Summer**: Normal (0.28,0.08) **Winter/ Spring:** Normal (0.61,0.13)
MSC Process/*Salmonella* spp.					
Prescald Normal(Normal (0.52,0.64),1.31)	Postpick Normal(-1.98,1.12)	Prechill **Summer:** Normal (2.28,0.462) **Winter/Spring:** Normal (-1.35,0.92)	Postchill Normal (2.03,0.22)	Frames Pert (-3.29, -2.7,1.03)	MSC **Summer:** Uniform (0.189,2.1,051) **Winter/ Spring:** Triangular (-2.36,-1.7,1.997)
Ground Process/*Campylobacter* spp.					
Prescald **Winter/Summer:** Normal(Normal (5.24,0.47),0.80) **Spring:** Normal(Normal (3.42,0.88),1.36)	Postpick **Winter/Summer:** Triangular (1.76,1.76,6.39) **Spring:** Uniform(0.94,9.35)	Prechill Triangular (-1.79,2.34,2.83)	Postchill Normal (3.03,0.99)	Pregrind Beta-Binomial **< -1.00:** Normal (1.75,0.62) **> -1.00:** Uniform (1.8,3.0)	Ground Product Normal (2.00,0.92)
Ground Process/*Salmonella* spp.					
Prescald Normal(Normal (-0.945,.54),1.44))	Postpick Normal(-1.72,1.81)	Prechill Normal (-2.41,1.62)	Postchill Normal (-2.5,0.46)	Pregrind Normal (1.56,0.87)	Ground Normal (-1.55,0.80)

Table 3.4 Reduction Distributions by Pathway
Distributions of the mean reduction from the prior sampling location.

Parts Process/*Campylobacter* spp. Reduction				
Postpick **Summer:** Normal (-1.59,0.15) **Winter/Spring:** Normal (-2.57,0.18)	Prechill **Summer:** Normal (-1.35,0.11) **Winter/Spring:** (-2.41,0.18)	Postchill **Summer:** Normal (-4.04,0.10) **Winter/Spring:** Normal (-2.00,0.13)	Drums No change	Breast No change
Parts Process/*Salmonella* spp. Reduction				
Postpick **Summer:** Normal (-2.56,0.17) **Winter/Spring:** Normal (-1.20,0.18)	Prechill **Summer:** Normal (-0.12,0.13) **Winter/Spring:** Normal (-0.65,0.22)	Postchill **Summer:** Normal (-0.67,0.09) **Winter/Spring:** Normal (-0.22,0.40)	Drums Normal (-0.78,0.05)	Breast Normal(-0.09,0.07)
MSC Process/*Campylobacter* spp. Reduction				
Postpick Normal(-2.14,0.20)	Prechill **Summer:** Normal (-0.44,0.14) **Winter/Spring:** Normal (-2.20,0.24)	Postchill **Summer:** Normal (-3.64,0.53) **Winter/Spring:** Normal (-1.88,0.21)	Frames Normal(0.28,0.07)	MSC **Summer:** Normal (-0.28,0.08) **Winter/Spring:** Normal(0.61,0.13)
MSC Process/*Salmonella* spp. Reduction				
Postpick Normal(-2.50,0.18)	Prechill **Summer:** Normal(0.64,0.15) **Winter/Spring:** Normal(-0.30,0.12)	Postchill **Summer:** Normal (-0.69,0.10) **Winter/Spring:** Normal(0.25,0.05)	Frames Normal (-0.22,0.06)	MSC **Summer:** Normal(1.66,0.11) **Winter/Spring:** Normal(3.20,0.09)
Ground Process/*Campylobacter* spp. Reduction				
Postpick **Winter/Summer:** Normal (-1.94,0.14) **Spring:** Normal(0.02,0.22)	Prechill **Winter/Summer:** Normal (-2.17,0.15) **Spring:** Normal(-2.31,0.18)	Postchill Normal(-4.16,0.14)	Pregrind Normal(2.10,0.19)	Ground Normal(-1.08, 0.2)
Ground Process/*Salmonella* spp. Reduction				
Postpick Normal(-0.78,0.24)	Prechill Normal(-0.69,0.24)	Postchill Normal(-0.09,0.17)	Pregrind Normal(0.95,0.10)	Ground Normal(0,0.12)

3.3.7 Variability and Uncertainty

Variability in model building is the result of natural differentiation between samples within a population. Uncertainty (or error) is the variation that occurs as a result of unknown influences upon the variable parameters. Variability is expected in any process and the shape of the models attempts to define the distribution of that variability (Nauta et al., 2005). Uncertainty

is a lack of knowledge with regard to parametric estimations and must be addressed in the model so that decisions are made with knowledge of factors that may be influencing output (Vose, 2000). In general, variability can be reduced by further sampling and uncertainty may be decreased through a tighter rein on experimental design (Zwietering and Nauta, 2007).

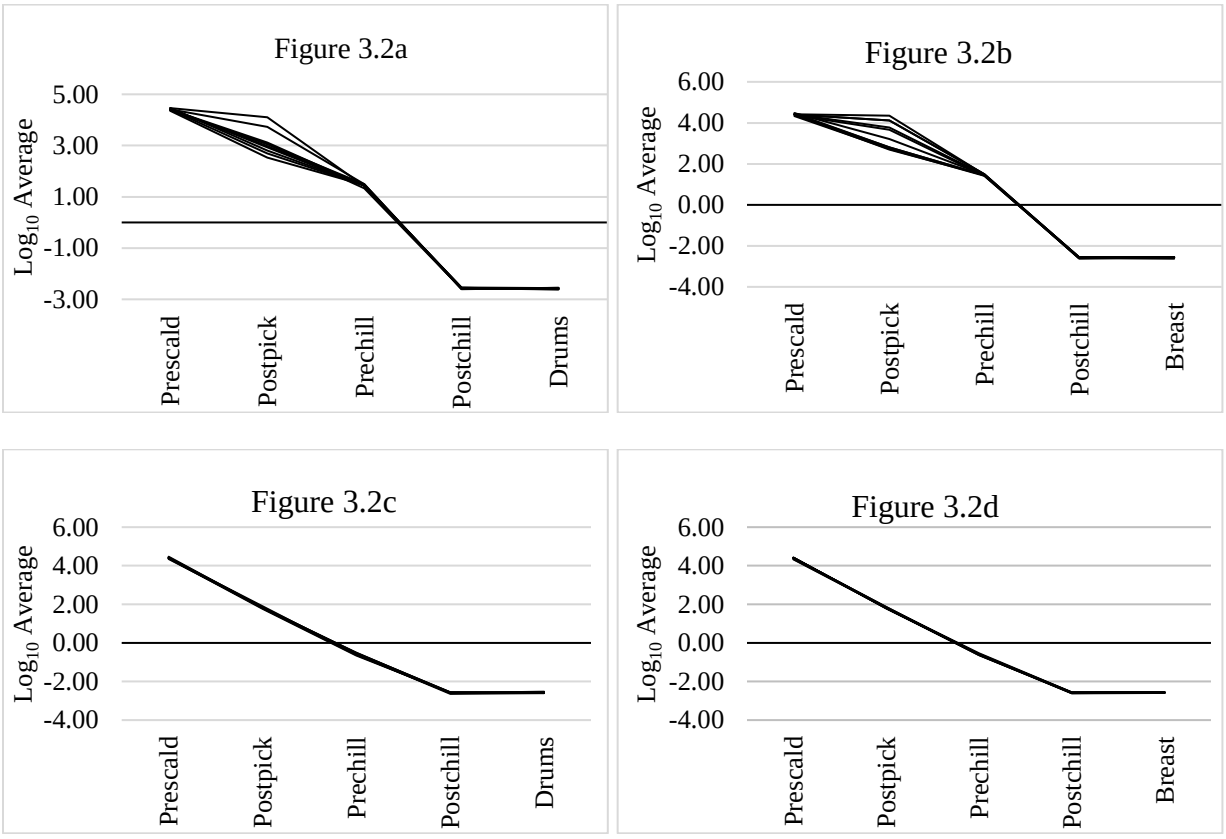
Variability is the natural, stochastic nature of the process, and is reflected in the models by way of distributions. Randomization was enacted with Monte Carlo simulations using random seed inputs, with each iteration representing a different flock. Between-flock variability was exhibited for each model by adjusting the incoming node (pre-scald) through the use of a lognormal distribution in which the mean was variable, based upon a lognormal distribution of the overall mean and the mean of the individual flock standard deviations, with the variation being represented by the overall node standard deviation.

Measurement uncertainty was included into the models by running 100 simulations of the original distributions, from which the means for each simulation were recorded and input into the final simulations as respective distribution parameters. This allowed the models to incorporate both variation and uncertainty in the simulations, while still allowing for visualization of uncertainty on the location outputs (see Figures 3.2a- 3.2o. Separating these two types of error is important to target and lower uncertainty in future analyses Gougouli and Koutsoumakis, 2015; Vose, 2000), and can prevent confusion between them (Pouillot and Delignette-Miller, 2010).

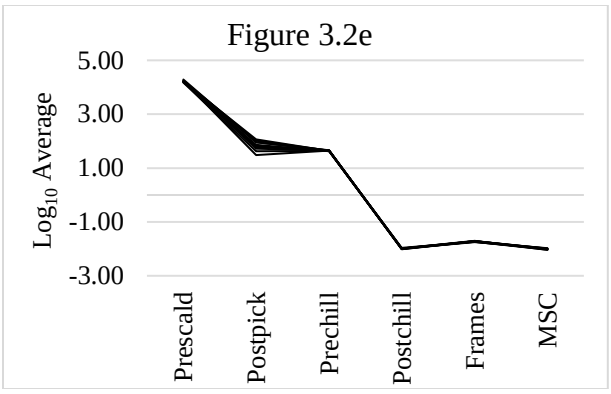
3.4 *Campylobacter* spp. and *Salmonella* spp. Model Results

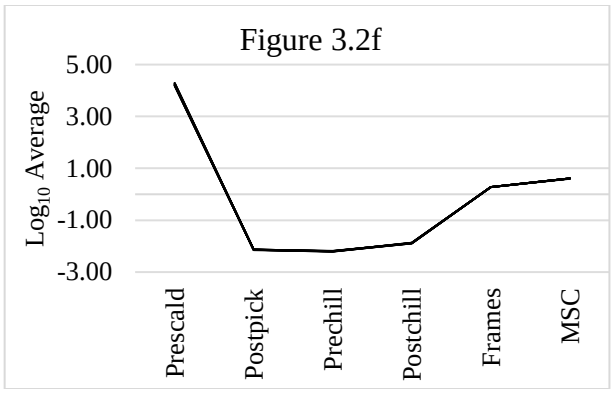
From 100 simulations with 1000 iterations, 10 simulation pathways were randomly chosen and charted for each product pathway (Figures 3.2a-3.2o). The *Campylobacter* spp. pathway revealed that there was very little measurement uncertainty in the winter drum and breast processes. The only node at which parameter uncertainty was evident was at the postpick

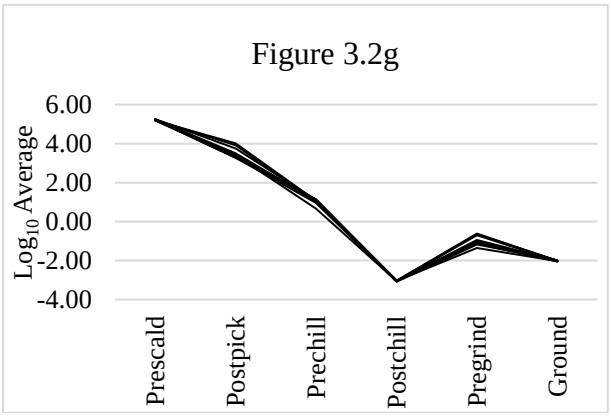
location for the summer models. For the MSC winter/spring process, there was not an evident amount of measurement uncertainty in the chosen simulations, and there was only a small

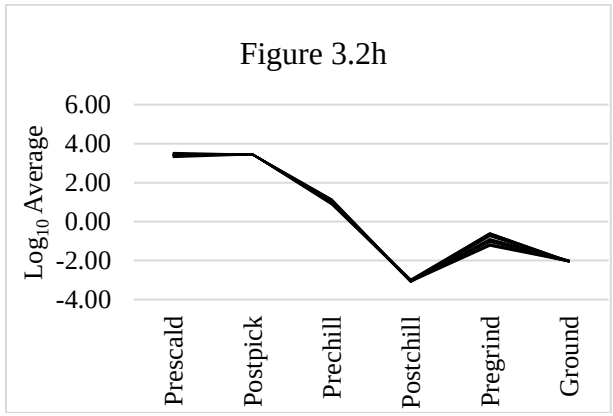


Figures 3.2a- 3.2d Mean Log_{10} Counts by Location and Pathogen Type
10 random samples taken from population (100 simulations with 1000 iterations). Each chart represents a specific process, with variation within each node indicating uncertainty in the model at that location.
[a]Mean Log_{10} Drum Flow for *Campylobacter* spp.by Location/Summer
[b]Mean Log_{10} Breast Flow for *Campylobacter* spp. by Location/Summer
[c]Mean Log_{10} Drum Flow for *Campylobacter* spp. by Location/Winter-Spring
[d]Mean Log_{10} Breast Flow for *Campylobacter* spp. by Location/Winter-Spring

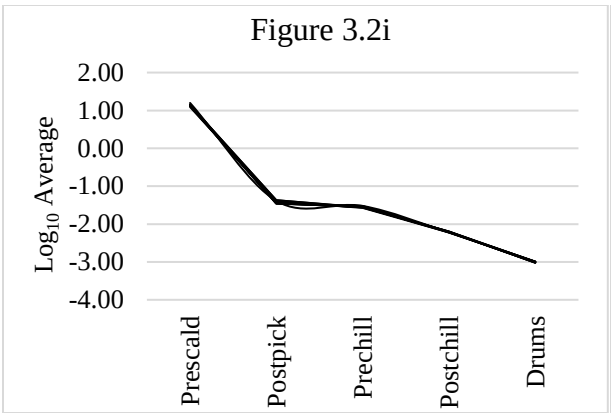


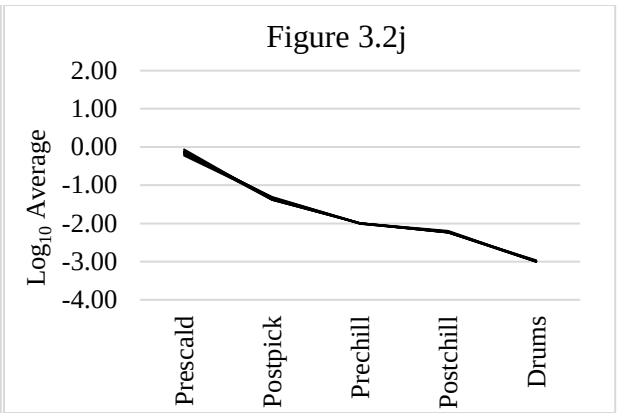


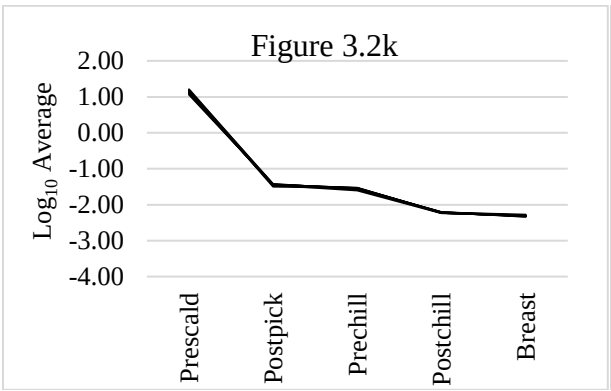


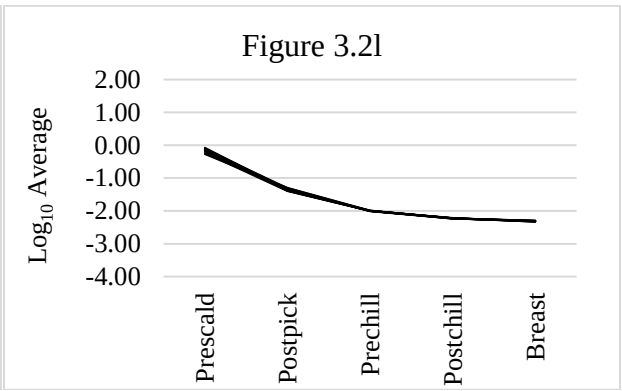


Figures 3.2e-3.2h Mean Log_{10} Counts by Location and Pathogen Type (cont.)
10 random samples taken from population (100 simulations with 1000 iterations). Each chart represents a specific process, with variation within each node indicating uncertainty in the model at that location.
[e]Mean Log_{10} MSC Flow for *Campylobacter* spp. by Location/Summer
[f]Mean Log_{10} MSC Flow for *Campylobacter* spp. by Location/Winter-Spring
[g]Mean Log_{10} Ground Flow for *Campylobacter* spp. by Location/Winter-Summer
[h] Mean Log_{10} Ground Flow for *Campylobacter* spp. by Location/Spring

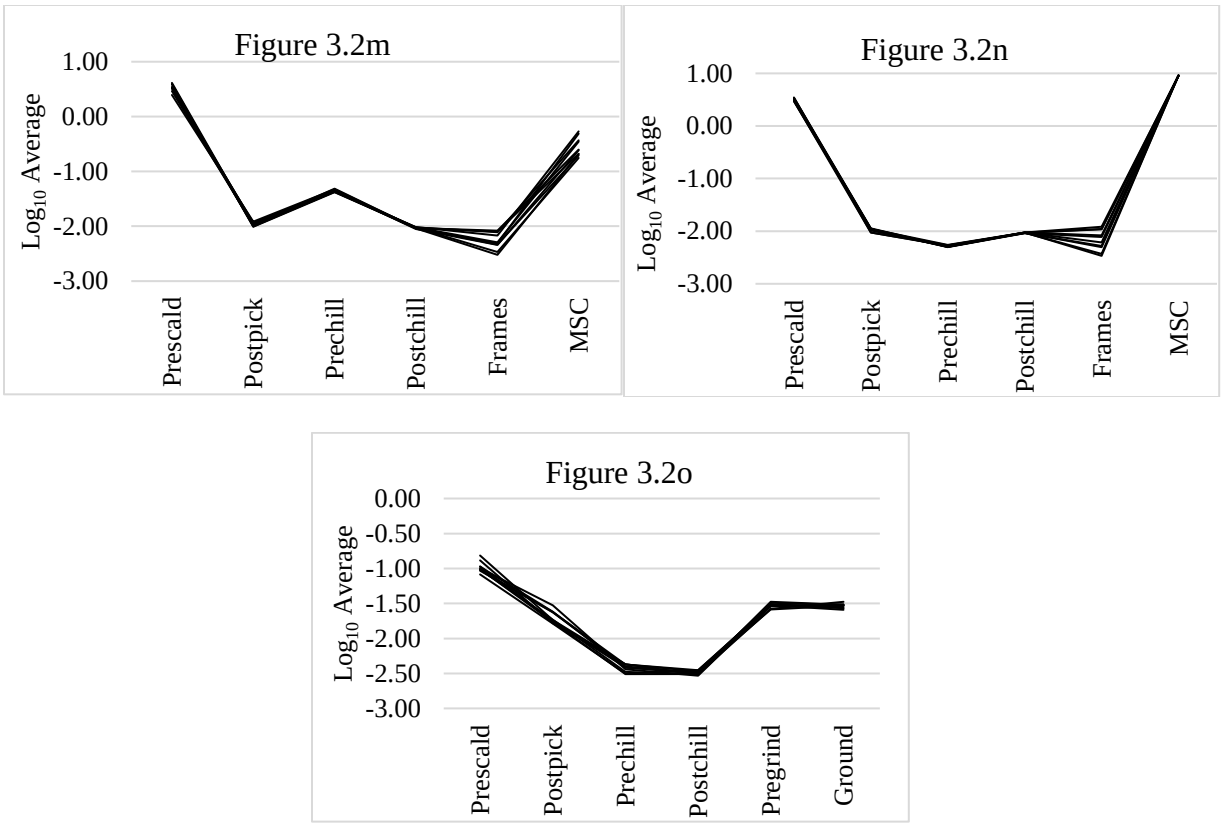








Figures 3.2i-3.2l Mean Log_{10} Counts by Location and Pathogen Type (cont.)
10 random samples taken from population (100 simulations with 1000 iterations). Each chart represents a specific process, with variation within each node indicating uncertainty in the model at that location.
[I] Mean Log_{10} Drum Flow for *Salmonella* spp. by Location/Summer
[j] Mean Log_{10} Drum Flow for *Salmonella* spp. by Location/Winter-Spring
[k] Mean Log_{10} Breast Flow for *Salmonella* spp. by Location/Summer
[l] Mean Log_{10} Breast Flow for *Salmonella* spp.by Location/Winter-Spring



Figures 3.2m-3.2o Mean Log_{10} Counts by Location and Pathogen Type (cont.)
10 random samples taken from population (100 simulations with 1000 iterations). Each chart represents a specific process, with variation within each node indicating uncertainty in the model at that location.
[m] Mean Log_{10} MSC Flow for *Salmonella* spp. by Location/Summer
[n] Mean Log_{10} MSC Flow for *Salmonella* spp. by Location/Winter-Spring
[o] Mean Log_{10} Ground Flow for *Salmonella* spp.by Location

amount visible at the postpick location for the summer MSC pathway. The ground process was separated differently by season, with the winter/summer pathway exhibiting measurement variability at the postpick, prechill, and pregrind locations. The spring pathway appears to have the least variation at postpick, and the greatest at pregrind.

For *Salmonella* spp. parts pathways there was very little variation, although a small amount was evident in the summer drums pathway at the prescald and postpick locations. The winter/spring flow revealed a small amount of measurement uncertainty in the prescald node. For

the breast product, the *Salmonella* spp. means by node reveal slight variation at the prescald location for the winter/spring pathway. There was a good deal more uncertainty between the mean *Salmonella* spp. values for the frame location for both the summer and winter models, with the variation still evident for the MSC product at the end of the summer flow. There was some variation for prescald and postpick locations for the *Salmonella* spp. flow for the ground product, but neither exhibited a significant difference between seasonal pathways ($p \leq 0.05$).

3.4.1 Pathogen Pathway Outputs

For illustrative purposes, the output for simulation #10 (out of the 100 simulations) was chosen for each cumulative display (Figures 3.3a-3.3f). The *Campylobacter* spp. parts pathway resulted in identical output for the drum and breast product, so these results were combined. Both the summer and winter seasonal output for both part types resulted in approximately 9% positive samples at the endpoints. This result was actually quite close to the performance standards established by USDA at 7.7%. The MSC models resulted in a 25.2% positive *Campylobacter* spp. rate in the summer and a 47.4% rate in the winter, a much higher positive rate than that predicated by the USDA pathogen standards. For ground *Campylobacter* spp. product, the simulations resulted in a 15.8% frequency of positives during the Summer/Winter seasons, and a 30.7% rate of positive *Campylobacter* spp. occurrence during the spring process simulation.

For *Salmonella* spp. there were 17.5% positive results in the simulation outputs for the drum product during summer and 17.6% for the winter. The summer breast product was at a positive frequency of 32.8% and the winter/spring at 31.9% at output. For the MSC flow, there was a 77% rate of *Salmonella* spp. positives at output for the summer model, and a 96%

frequency of *Salmonella* spp. positives for the winter/spring output. The ground product model (all seasons) exhibited a distribution with 55% positive *Salmonella* spp. samples at output.

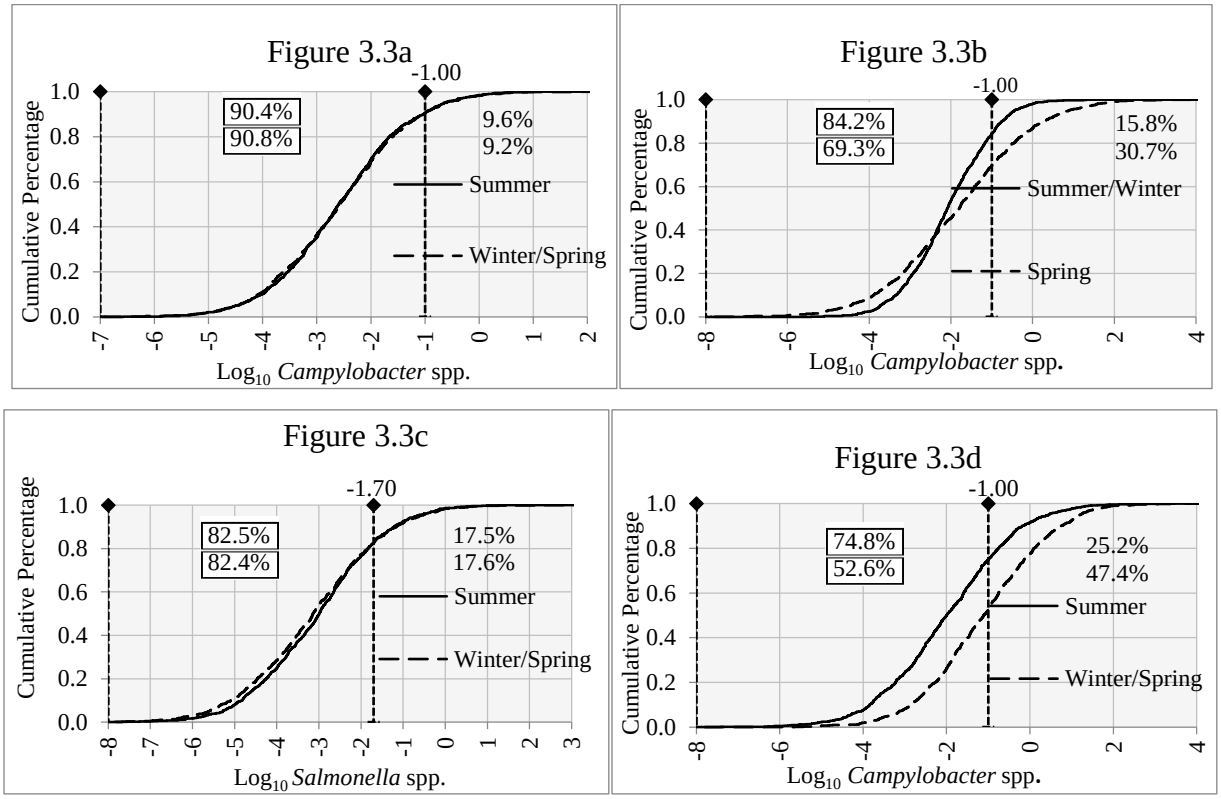


Figure 3.3a-3.3d Cumulative Distribution Output by Part/Season Pathway
Each output displays simulation #10 from 100 simulations/1000 iterations. Line at -1.00 for *Campylobacter* and -1.70 for *Salmonella* designates the cut-off for a positive result. The percentages in the white box represent the expected frequency of negative results and the percentages on the right represent the expected percent positive.
[a] Final Output Parts/*Campylobacte*r spp.
[b] Final Output Ground/*Campylobacter* spp.
[c] Final Output Drums/*Salmonella* spp.
[d] Final Output MSC/*Campylobacter* spp.

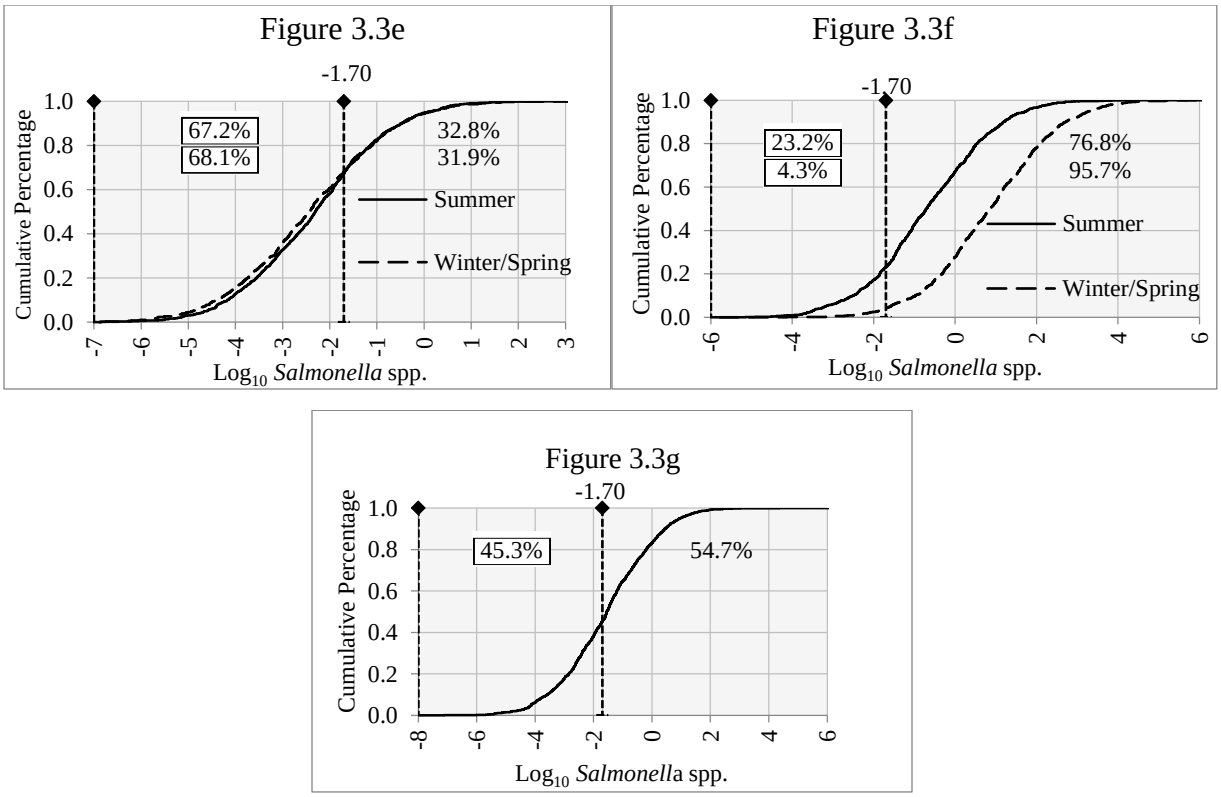


Figure 3.3e-3.3h Cumulative Distribution Output by Part/Season Pathway
Each output displays simulation #10 from 100 simulations/1000 iterations. Line at -1.00 for *Campylobacter* and -1.70 for *Salmonella* designates the cut-off for a positive result. The percentages in the white box represent the expected frequency of negative results and the percentages on the right represent the expected percent positive.
[e] Final Output Breast/*Salmonella* spp.
[f] Final Output MSC/*Salmonella* spp.
[g] Final Output Ground/*Salmonella* spp.

3.4.1.1 Goodness of Fit for Simulation Outputs

The Chi square goodness of fit test was used to compare the simulation output results to that of the final product results from the raw data. The comparisons were made with binned data, with the frequency data within each bin being compared. Bins were of equal size with the exception of the frequency of values that resided below the LOD for both microbial classes,

which were handled as one single bin, regardless of size. With the following formula used to determine the Chi square score for each bin:

$$X^2 = \sum(O_i - E_i)^2 / E_i \text{ where;} \quad (3.4)$$

E_i is the expected frequency of positive samples for each bin, and O_i is the observed frequency of positive samples for each bin. The degrees of freedom value used to ascertain critical limit for each category was quantified as k-1, where, k is the number of bins used for comparison of the two distributions (see Table 3.5).

Table 3.5 Chi Square Goodness of Fit Results for *Campylobacter* spp. Model Outputs

Pathogen Type	Pathway	X^2 value
Campylobacter spp.	Parts	0.00 (All values for compared distributions were < -1.00)
	MSC (Winter/Spring) MSC (Summer)	0.12 0.48
	Ground (Winter/Summer) Ground (Spring)	0.03 0.19
Salmonella spp.	Drums (Summer) Drums (Winter/Spring) Breast (Summer) Breast (Winter/Spring)	0.23 0.25 0.11 0.02
	MSC (Winter/Spring) MSC (Summer)	0.06 0.33
	Ground (all seasons)	0.07

None of the pathways exhibited a significant difference between actual results at endpoint and output simulated data ($df = 5$, $p \leq 0.01$, t-crit = 0.554).

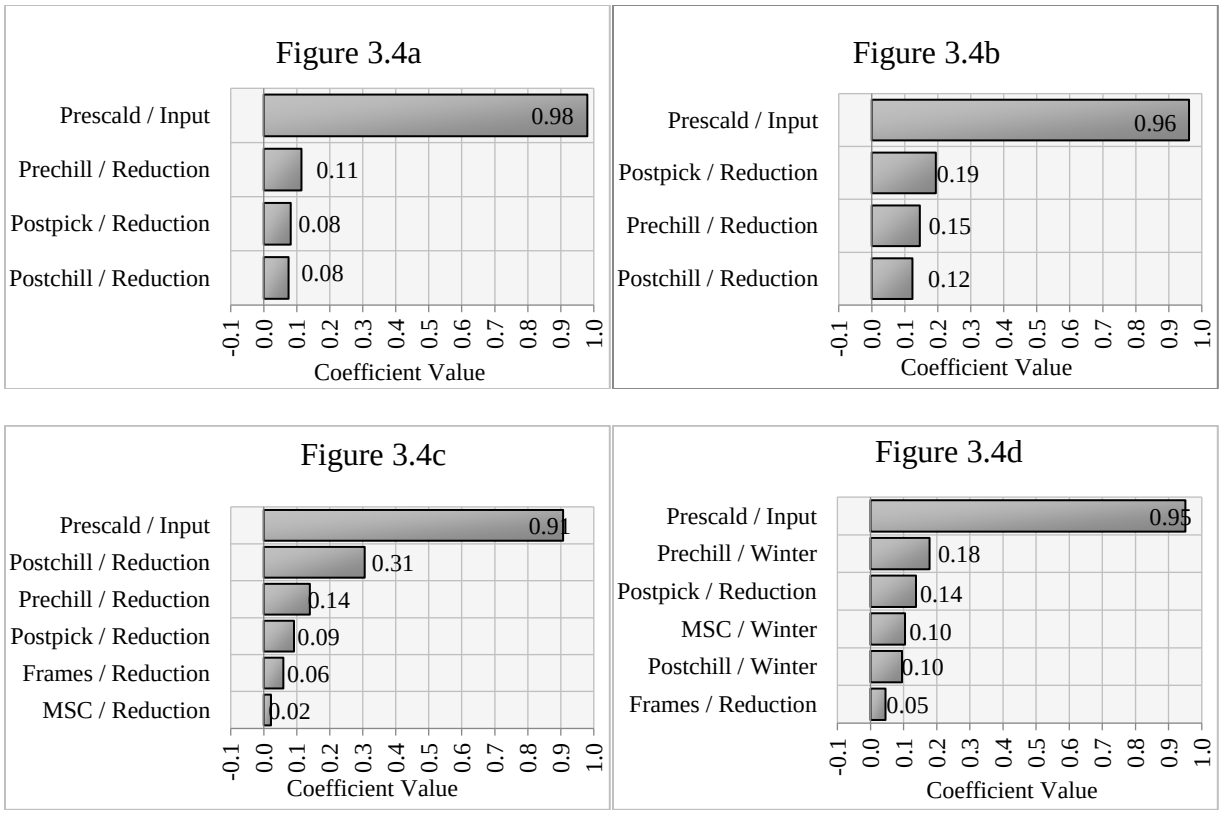
For each of the pathways, there was no significant difference between the frequencies of positive values by bin for the observed distribution (original fit) and expected distribution (final output of simulated data). These results indicate that the simulated data adequately represent the

actual collected data for frequency of positive values and, it can be expected that simulated reduction exercises for these pathways would also be representative of the actual process.

3.4.2. Sensitivity Analyses for Product Pathway by Pathogen Type

Sensitivity analyses were run for each pathway, with results showing the weight of each sample location (node) on the output distribution. In Figures 3.4a to 3.4m, the Spearman rank correlation coefficient for each node with relation to the model output is displayed. In all pathways, the prescald location was most influential upon the final pathogen distribution. followed by samples taken directly after the OLR cabinet, right before the chiller.

For the parts product summer flow, the location with the next highest weighting was the prechill node, whereas the postpick location had the most influence during the winter-spring season. The MSC product showed the second most beneficial location of reduction to be at postchill during the summer, whereas the prechill node was the best spot for reduction in the winter/spring pathway. The ground product output was most influenced by prescald for both the winter/summer and spring pathways. However, as this analysis was an attempt to discover the most beneficial places to reduce counts **before** production end, the next highest scoring location, pregrind for winter/spring, and postpick for summer, was used for reduction simulations.



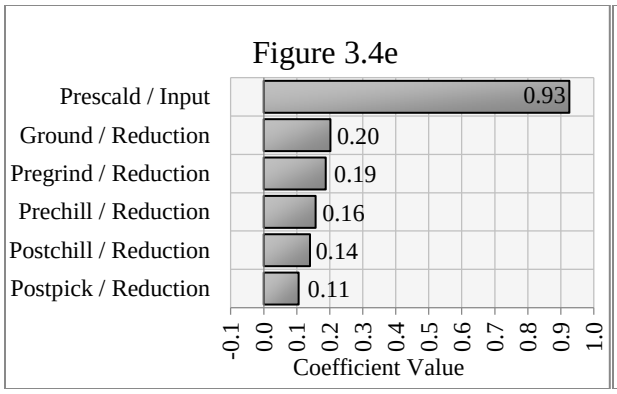
Figures 3.4a-3.4d Sensitivity Analyses by Pathogen Type and Pathway

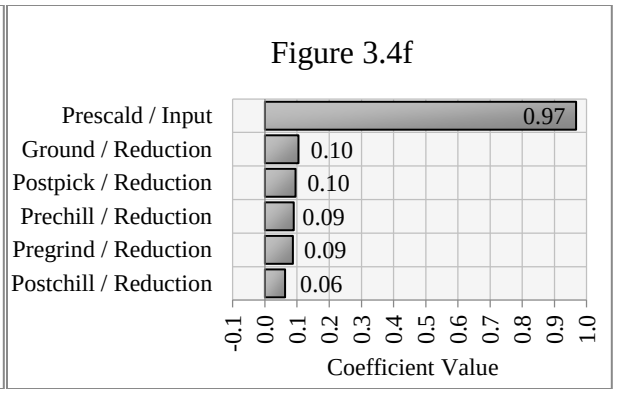
[a] Spearman Rank Correlation for *Campylobacter* spp. Parts Process (all)/Summer

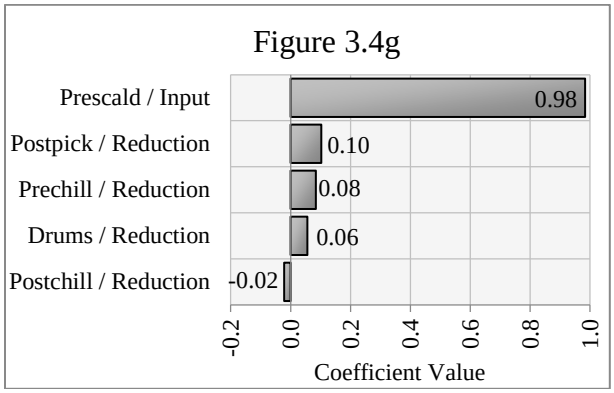
[b] Spearman Rank Correlation for *Campylobacter* spp. Parts Process (all)/Winter-Spring

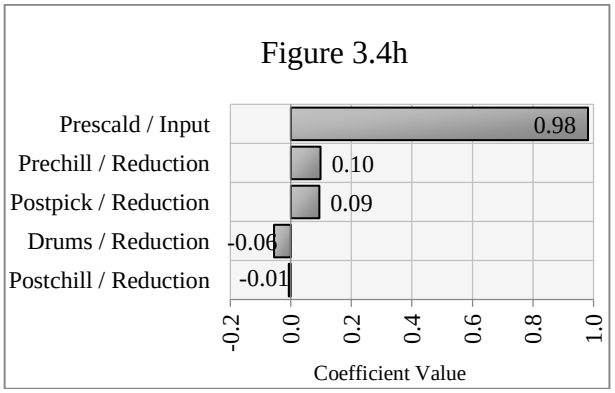
[c] Spearman Rank Correlation for *Campylobacter* spp. MSC Process/Summer

[d] Spearman Rank Correlation for *Campylobacter* spp. MSC Process/Winter-Spring









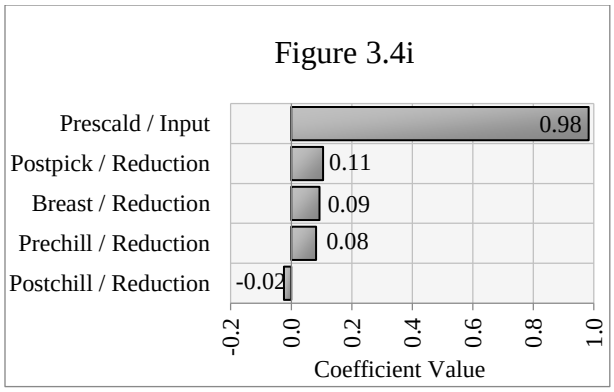
Figures 3.4e-3.4h Sensitivity Analyses by Pathogen Type and Pathway

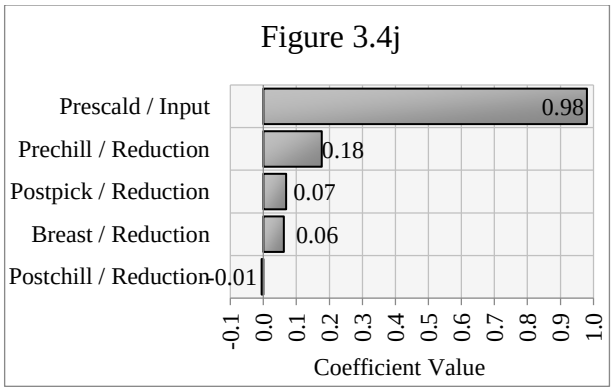
[e] Spearman Rank Correlation for *Campylobacter* spp. Ground Process/Winter-Summer

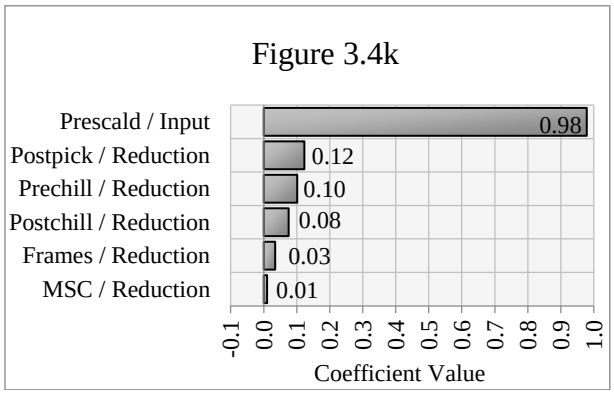
[f] Spearman Rank Correlation for *Campylobacter* spp. Ground Process/Spring

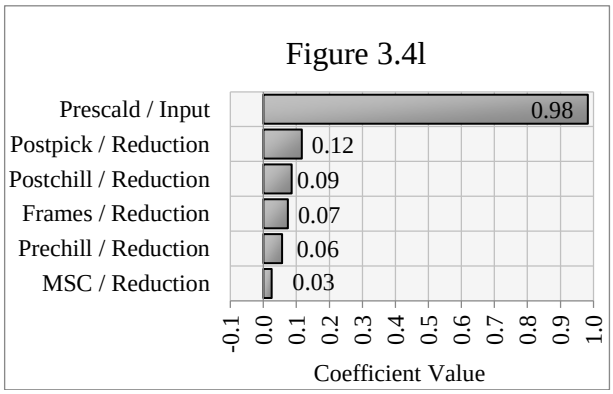
[g] Spearman Rank Correlation for *Salmonella* spp. Drums Process/Summer

[h] Spearman Rank Correlation for *Salmonella* spp.Drums Process/Winter-Spring









Figures 3.4i-3.4l Sensitivity Analyses by Pathogen Type and Pathway

[i] Spearman Rank Correlation for *Salmonella* spp. Breast Process/Summer

[j] Spearman Rank Correlation for *Salmonella* spp. Breast Process/Winter-Spring

[k] Spearman Rank Correlation for *Salmonella* spp. MSC Process/Summer

[l] Spearman Rank Correlation for *Salmonella* spp. MSC Process/Winter-Spring

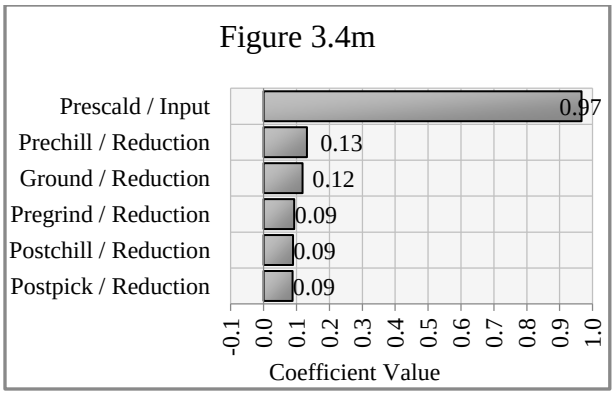


Figure 3.4m Sensitivity Analyses by Pathogen Type and Pathway
[m] Spearman Rank correlation for *Salmonella* spp. Ground Process

For all product types and pathways the prescald location most significantly influenced the endpoint product *Salmonella* spp. distribution. The drums and breast product both exhibited a secondary influence of the postpick node for the summer months and for the prechill node during the winter/spring season. The MSC product pathway for both the summer and winter/spring seasons was most heavily influence (after the prescald node) by the postpick location distribution. For the ground product, the prechill was the second weightiest influencer of endpoint results for *Salmonella* spp. load.

3.4.3 Simulated Reductions for Campylobacter spp. Models

Based on the results of the sensitivity analyses, three simulated reductions for the two most heavily weighted locations were applied to the models. For each of the three simulations, 1000 iterations were processed using a Monte Carlo Latin Hypercube algorithm. Final simulations were chosen based upon the most beneficial reductions to arrive at or below the pathogen frequency allowances set by the USDA.

For the simulations, reduction distributions were developed that were to represent the changes that occurred between each node. Each reduction was normally distributed with the

mean being the mean reduction being a reasonable choice, subjectively based upon the current distributions, with the standard deviation being the mean standard deviation of the 100 simulated reductions from the original model. This assumed that the model variation would be the same for the simulated model as it was for the actual model.

For the parts product, the *Campylobacter* spp. drum and breast pathways were combined as there was not a statistically significant difference between the two outputs. The summer and winter parts model reductions were isolated to the prescald locations as these simulations brought the levels of *Campylobacter* spp. in the simulated product distributions to levels well within the USDA performance parameters. Additional reductions of between 1.75

And 2.25 $\log_{10}$ resulted in *Campylobacter* spp. frequencies of 0.4% to 1.8% for summer and 0.8% to 1.6% for winter produced parts product (Figures 3.5a-3.5m).

The *Campylobacter* spp. models for MSC required additional reductions from 1.50 to 2.00 log prescald for the summer pathways and from 2.00 to 2.50 for the winter pathways, along with postchill mean reductions of 1.00 log in the summer, and from 0.75 to 1.00 log means

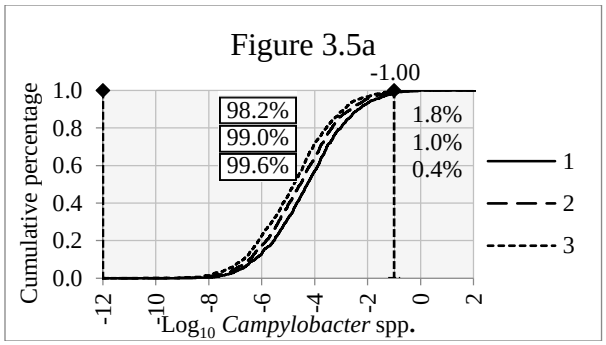
reductions at prechill during winter processing. For the summer/winter season ground product, additional reductions of 1.50 to 2.50 log at prescald and from 0.50 to 0.75 pregrind resulted in a frequency of *Campylobacter* spp. positives from 0.4% to 1.0%, whereas extra reductions of 3.00 log at prescald and from 2.00 to 2.50 log at postpick resulted in MSC outputs exhibiting 1.3% to 0.8% *Campylobacter* spp. positive carcasses during the winter pathway.

The *Salmonella* spp. parts simulations with additional reductions of 0.75 to 1.25 prescald and 0.50 postpick for both the summer and winter/spring drums pathways resulted in output frequency positives from 3.7 to 1.4% and from 5.0% to 2.6%, respectively. For the breast

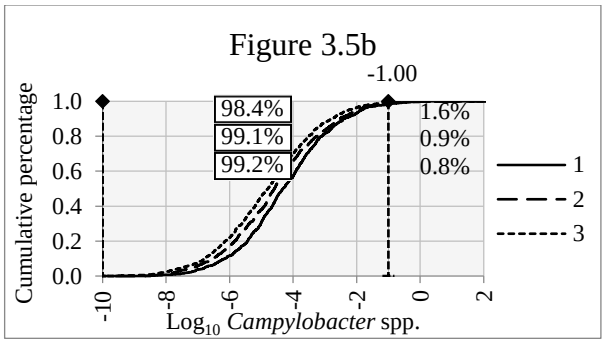
product simulations, adding reductions of 1.00 to 1.75 prescald, 0.50 postpick (summer) and 0.50 prechill (winter/spring) ended in output distributions reflecting 3.3 to 7.0% positive results for the summer months and 3.2 to 6.1% positive for the winter/spring season.

The summer model for the MSC product required additional mean reductions of 2.00 to 2.75 $\log_{10}$ prescald and then another 0.75 $\log_{10}$ reduction postpick to meet the USDA standards with output of *Salmonella* spp. frequency between 11.46% to 20.02% positive. The winter/spring pathway required even higher added reductions from 2.50 to 3.00 $\log_{10}$ prescald and 1.50 $\log_{10}$ postpick to arrive at positive frequencies quite close to the established parameters (18.21% to 24.27%).

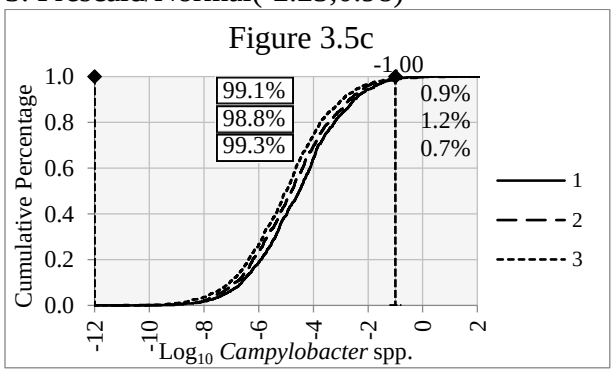
All seasons were modeled together for the *Salmonella* spp. load on ground product as there was no significant difference between these samples ($p \leq 0.05$). Additional reductions were only needed at the prescald node to achieve simulated output within required parameters. Mean reductions from 1.25 to 2.00 resulted in simulation output for ground product at 11.8% to 24.0% positive for *Salmonella* spp.



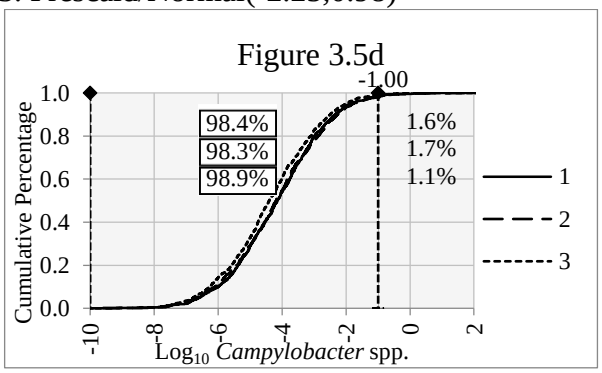
1. Prescald/Normal(-1.75,0.98)
2. Prescald/Normal(-2.00,0.98
3. Prescald/Normal(-2.25,0.98)



1. Prescald/Normal(-1.75,0.98)
2. Prescald/Normal(-2.00,0.98)
3. Prescald/Normal(-2.25,0.98)



1.Prescald/Normal(-1.50,0.20)
Postchill/Normal(-1.00,0.53)
2. Prescald/Normal(-1.75,0.20)
Postchill/Normal(-1.00,0.53)
3. Prescald/Normal(-2.00,0.20)
Postchill/Normal(-1.00,0.53)



1.Prescald/Normal(-2.00,0.20)
Prechill/Normal(-1.00,0.21)
2.Prescald/Normal(-2.25,0.20)
Prechill/Normal(-1.00,0.21)
3.Prescald/Normal(-2.50,0.20)
Prechill/Normal(-1.00,0.21)

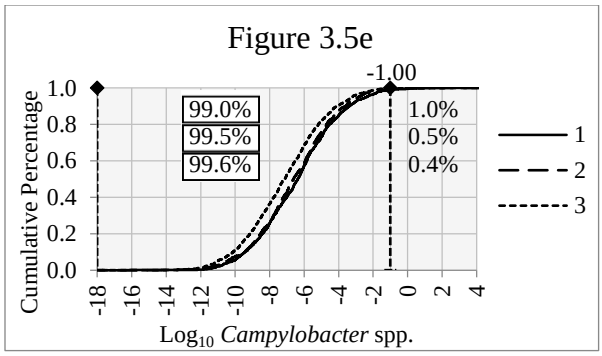
Figure 3.5a-3.5d Results of Simulated Reduction Models by Pathogen and Product Pathway Line at -1.00 for *Campylobacter* and -1.70 for *Salmonella* designates the cut-off for a positive result. The percentages in the white box represent the expected frequency of negative results and the percentages on the right represent the expected percent positive.

[a] Summer Parts Simulations/*Campylobacter* spp.

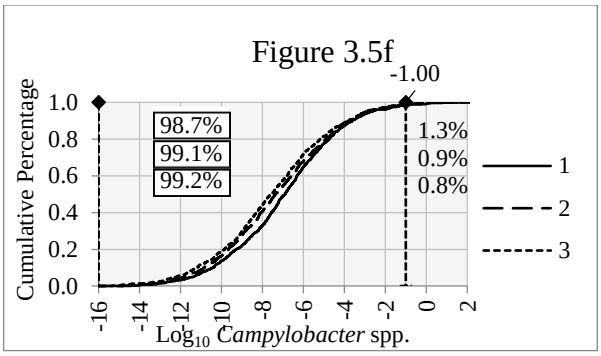
[b] Winter/Spring Parts Simulations/*Campylobacter* spp

[c] Summer MSC Simulations/*Campylobacter* spp.

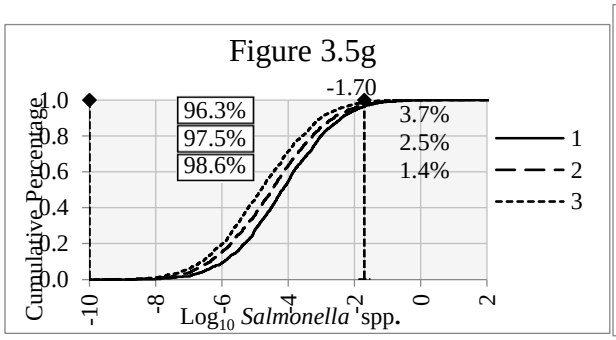
[d] Winter/Spring MSC Simulations/*Campylobacter* spp.



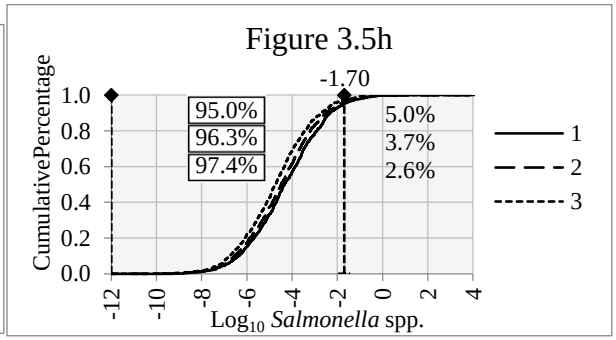
1.Prescald/Normal(-1.50,1.50)
Pregrind/Normal(-0.75,1.50)
2 Prescald/Normal(-2.00,1.50)
Pregrind/Normal(-0.50,1.50)
3.Prescald/Normal(-2.50,1.50)
Pregrind/Normal(-0.50,1.50)



1. Prescald/Normal(-3.00,1.50)
Postpick/Normal(-2.00,1.50)
2. Prescald/Normal(-3.00,1.50)
Postpick/Normal(-2.25,1.50)
3. Prescald/Normal(-3.00,1.50)
Postpick/Normal(-2.50,1.50)



1.Prescald/Normal(-0.75,1.42)
Postpick/Normall(-0.50,0.30)
2.Prescald/Normal(-1.00,1.42)
Postpick/Normal(-0.50,0.30)
3.Prescald/Normal(-1.25,1.42)
Postpick/Normal(-0.50,0.30)



1.Prescald/Normal(-0.75,1.42)
Prechill/Normal(-0.50,0.30)
2.Prescald/Normal(-1.00,1.42)
Prechill/Normal(-0.50,0.30)
3.Prescald/Normal(-1.25,1.42)
Prechill/Normal(-0.50,0.30)

Figure 3.5e-3.5h Results of Simulated Reduction Models by Pathogen and Product Pathway (cont.) Line at -1.00 for *Campylobacter* and -1.70 for *Salmonella* designates the cut-off for a positive result. The percentages in the white box represent the expected frequency of negative results and the percentages on the right represent the expected percent positive.

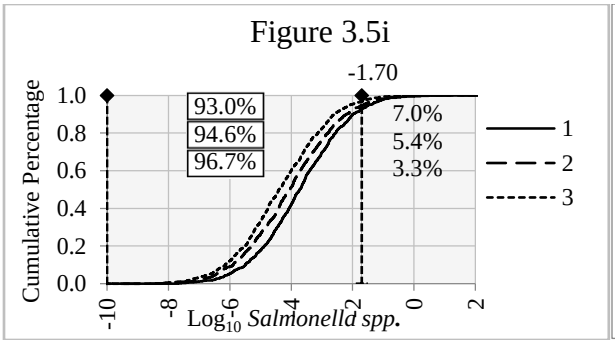
[e] Summer/Winter Ground Simulations/*Campylobacter* spp.

[f] Spring Ground Simulations/*Campylobacter* spp.

[g] Summer Drum Simulations/*Salmonella* spp.

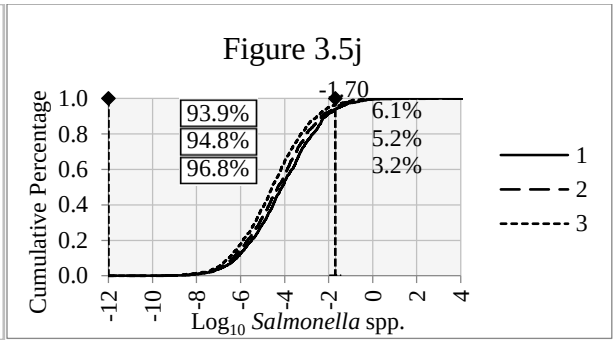
[h] Winter/Spring Drum Simulations/*Salmonella* spp.

Figure 3.5i



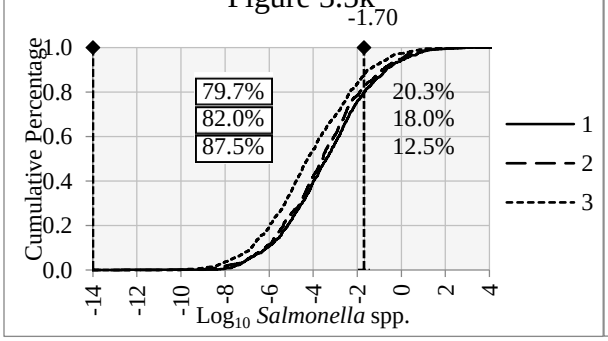
1.Prescald/Normal(-1.00,1.42)
Postpick/Normal(-0.50,0.30)
2.Prescald/Normal(-1.25,1.42)
Postpick/Normal(-0.50,0.30)
3.Prescald/Normal(-1.50,1.42)
Postpick/Normal(-0.50,0.30)

Figure 3.5j



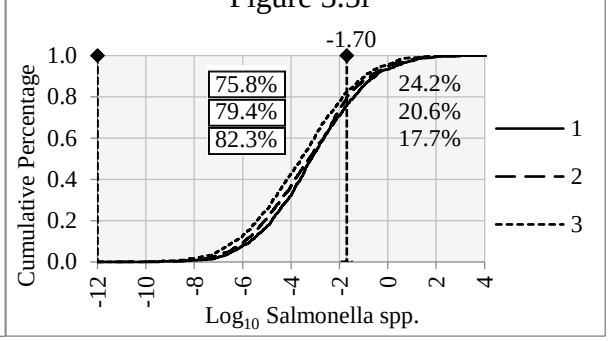
1.Prescald/Normal(-1.25,1.42)
Prechill/Normal(-0.50,0.30)
2.Prescald/Normal(-1.50,1.42)
Prechill/Normal(-0.50,0.30)
3.Prescald/Normal(-1.75,1.42)
Prechill/Normal(-0.50,0.30)

Figure 3.5k



1.Prescald/Normal(-2.00,1.45)
Postpick/Normal(-0.75,0.18)
2.Prescald/Normal(-2.25,1.45)
Postpick/Normal(-0.75,0.18)
3.Prescald/Normal(-2.75,1.45)
Postpick/Normal(-0.75,0.18)

Figure 3.5l



1.Prescald/Normal(-2.50,1.45)
Postpick/Normal(-1.50,0.18)
2.Prescald/Normal(-2.75,1.45)
Postpick/Normal(-1.50,0.18)
3.Prescald/Normal(-3.00,1.45)
Postpick/Normal(-1.50,0.18)

Figure 3.5i-3.5l (cont.) Results of Simulated Reduction Models by Pathogen and Product Pathway (cont.)

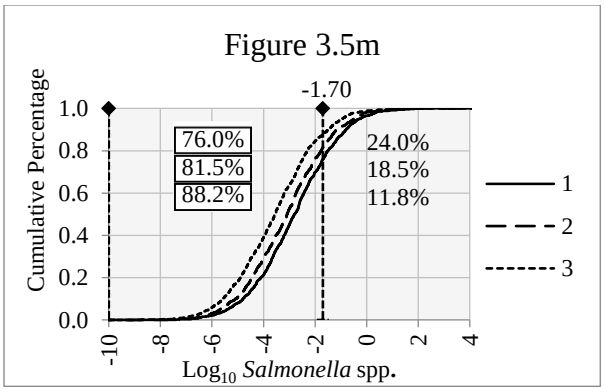
Line at -1.00 for *Campylobacter* and -1.70 for *Salmonella* designates the cut-off for a positive result. The percentages in the white box represent the expected frequency of negative results and the percentages on the right represent the expected percent positive.

[i] Summer Breast Simulations/*Salmonella* spp.

[j] Winter Breast Simulations/*Salmonella* spp

[k] Summer MSC Simulations/*Salmonella* spp.

[l] Winter/Spring MSC Simulations/*Salmonella* spp.



1.Prescald/Normal(-1.25,1.50)
2.Prescald/Normal(-1.50,1.50)
3.Prescald/Normal(-2.00,1.50)

Figure 3.5m (cont.) Results of Simulated Reduction Models by Pathogen and Product Pathway

Line at -1.00 for *Campylobacter* and -1.70 for *Salmonella* designates the cut-off for a positive result. The percentages in the white box represent the expected frequency of negative results and the percentages on the right represent the expected percent positive.

[m] Ground Simulations/*Salmonella* spp.

3.5 Conclusion

Fit for all the models was appropriate, given that the "negative" (<-1.00 for *Campylobacter* and <-1.70 for *Salmonella*) bins were larger for all models than all other bins. Results for all pathogen and product pathways were expressed with cumulative distribution outputs of predicted product distributions (Figures 3.3a-3.3f). Given these results, none of the products would have met USDA pathogen performance standards for either *Campylobacter* spp. or *Salmonella* spp. standards, despite the fact that the counts were consistently reduced throughout the processes (with the exception of the slight increase seen at the grinder and frames for ground and MSC product, respectively).

Sensitivity analyses (Figures 3.4a-3.4m) indicate reductions taken at the prescald location would be the most advantageous to lowering pathogen load in the final product, pointing to the importance of mitigation strategies at grow-out (Berghaus et al., 2013).

Results from the models were illustrated by distributions incorporating a wide range of possible values. Reportable counts were for a 1 gram sample and the models were representative of this. However, extrapolation of these results to incorporate the actual risk of consumption should incorporate the predicted serving size, for instance, a 100 gram sample.

3.5.1. Campylobacter spp.

The parts pathways for *Campylobacter* spp. are similar by season for both parts types in that they decrease up to the end of the process, where counts plateau. The MSC summer season product pathway exhibits a decrease at postpick, does not decrease again until postchill and then evens off at this point. For the winter/spring model the mean *Campylobacter* spp. load goes down at the postpick location and does not decrease again, instead increasing at the frame location and again in the final product.

The parts models for *Campylobacter* spp. reveal outputs similar to the USDA FSIS pathogen standards for all seasons, but the MSC product had a much higher frequency of *Campylobacter* spp. positive results in the winter/spring season than for the summer. The ground product model for *Campylobacter* spp. for winter/summer revealed reductions through the process to postchill, and then increased at pregrind, with some decrease once it arrived to the ground stage. For the spring product there was less of a load decrease at postpick, with a larger reduction at prechill and again at postchill, but then increased at pregrind, with a small decrease at the end of the process.

Overall the frequency of positive results for *Campylobacter* spp. were higher in the spring season for ground product and for winter/spring seasons for both ground and MSC product. Models for the *Campylobacter* spp. pathways for all product types showed only slight

amounts of measurement uncertainty throughout the process, with the most being exhibited in the drum and breast parts product prescald and postpick nodes.

3.5.2. Salmonella spp.

For the *Salmonella* spp. parts pathway, the drums product goes down throughout the entire process for both seasons, with the breast product going down at postpick and plateauing at all other downstream locations. For MSC product counts go down postpick, but up prechill, going back down postchill and back up at the final product endpoint. The ground product goes down postpick and prechill and then back up during the pregrind process, leveling off at the final ground product.

Salmonella spp. loads at output for drum product were similar by season for drum and breast product, with the breast product having a higher pathogen load at endpoint. The MSC product had a much higher frequency of *Salmonella* spp. positive product for the winter/spring season, whereas the ground product had a similar load throughout the year. The most uncertainty for *Salmonella* spp. load was exhibited in the prescald and postpick nodes for the ground product pathway.

Reduction simulations resulted in expectations of output product that would sufficiently meet USDA-FSIS pathogen standards. However, the reductions needed to meet these standards can approximate quite large expectations of intervention capabilities and may not be reasonable- or quantifiable- in a processing environment.

At this juncture, a discussion of the level of reduction necessary to meet the USDA pathogen performance standards must be addressed. Although the simulated reductions required to meet the standards may be achieved with what appear to be reasonable reductions, these

reductions are additive to reductions already being achieved by the current process. For instance, in order to meet the performance standards required specified for *Salmonella* spp. load on MSC product, a facility would have to reduce the mean level on carcasses going into the picker by 4.50 to 5.25 $\log_{10}$ during the summer months and by 5.00 to 5.50 $\log_{10}$ during the winter. This is not an unsubstantial amount. Considering that the mean level on carcasses for each of these seasons is 0.52 log, these reductions appear unreasonable, not to mention unquantifiable at the processing level. This is somewhat due to the large amount of variation in the data (a standard deviation of 1.45 indicates that counts can range from -3.77 to 4.69 log at the 99% confidence interval), but the point remains that these levels of reduction may not be achievable in a processing environment.

Suggestions for further analyses would include a similar data collection where samples resulting in a negative lab result would be further enumerated in order to ascertain what percentage of negative results could be expected to be counts that were simply below the level of detection. Sampling where a larger number of rinses could be recovered at each node for each flock would also be beneficial in further refining the variation present at each location within a flock.

In addition, the fact that the incoming load (prescald) was the predominate factor influencing output distribution indicates that reducing counts before entering the production facility is essential for reduction of *Campylobacter* and *Salmonella* counts in parts, ground, and MSC poultry product. Data should be collected from live production on flocks before they enter the production facility in order to refine the reduction scenario that would result in the most desirable scenario at production endpoint.

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Chapter 4: Analysis of *Salmonella* and *Campylobacter* reduction by location in First processing: Targeting beneficial intervention application in poultry facilities

4.1 Abstract

Physical and chemical interventions during poultry processing may be used to reduce pathogen load on product. Although cidal agents may be used at almost every step in the processing flow, some application locations have proven to be more beneficial than others at reducing micro-organisms of interest at product endpoints. Isolating the chemical substances, and levels of these substances, at which the highest pathogen reductions occur may be used with existing pathogen reduction models to identify the most efficient means of controlling microbial levels during poultry processing.

Data at first processing locations from four poultry facilities were analyzed to determine the impact of intervention type, concentration and pH on $\log_{10}$ reduction for *Salmonella* spp. and *Campylobacter* spp. on product. Analysis of variance (ANOVA) tests for the impact of class variables on reduction, and multiple regression analyses by pH and ppm for each intervention were analyzed by *Salmonella* spp. or *Campylobacter* spp.

Results revealed line speed did not significantly affect pathogen reduction (although the *Salmonella* spp. reduction was statistically significant, the difference was quite small, a result of a high frequency of negative results). The presence of a post-pick cabinet resulted in a higher reduction for *Campylobacter* spp. and a lower reduction for *Salmonella* spp. samples than for samples without a cabinet present.

Using the results of ANOVA and multiple regression analyses, reductions were placed into existing models to infer reductions at specific locations during poultry processing. Use of these models may be beneficial in reducing pathogen load in poultry products.

4.2 Introduction

Campylobacter, one of the most common sources of foodborne illness (Stella et al., 2017) has been linked to consumption of poultry products (Crotta et al., 2017). *Campylobacter*, like most bacteria, likes warm temperatures, and a wet environment with a bit of food, provided by the organic matter left by poultry processing (Garcia-Sanchez et al., 2017). *Campylobacter* spp. has been associated with process events such as unsuccessful picking or venting, and poorly executed evisceration and lower scalding temperatures (Seliwiorstow et al., 2014).

Ingestion of *Campylobacter* doses between 2 to 4 $\log_{10}$ have been associated with infection and illness (Hunt et al., 2001). Symptoms include in diarrhea and/or vomiting and, in a few cases, become a precursor to Guillian-Barre syndrome, an autoimmune disorder that may further result in paralysis (Garcia-Sanchez et al., 2017).

There are not widely accepted methods of *Campylobacter* reduction at grow out (Ysunza and Le Ven, 2016). Researchers have found that *jejuni* was the most frequently recovered *Campylobacter* serotype at processing (Jones et al., 1991),interventions during processing have proven effective as cidal agents on poultry carcasses, although complete eradication of *Campylobacter* in poultry is not considered a reasonable expectation (Alter, 2017; Havelaar et al., 2007).

From the family *Enterobacteriaceae*, the genus *Salmonella* consists of many serotypes (approximately 2600), each which thrive in unique environments, making it difficult to target reductions for the entire group (De Cesare, 2018; Merino et al., 2017). Serotypes most commonly associated with poultry are Enteritidis, Hadar, Heidelberg, Infantis, Kentucky, Montevideo, Typhimurium, Swarzengrund, and Seftenberg (Narayan et al., 2017). Typhimurium

and Enteritidis are most frequently associated with human illness (Antunes et al., 2016). Although the most common serotype found in broiler processing was Kentucky, often comprising between 25-49% of samples taken (Finstad et al., 2012); it is not highly associated with human infection through consumption (Ricke et al., 2015).

Foodborne illnesses attributed to *Salmonella* spp. are estimated at approximately 1.2 million cases per year (CDC, 2018). The non-typhoidal form of *Salmonella* spp. is considered the source of about 35% of foodborne illnesses associated with poultry consumption (Nagel et al., 2013), and, as such, has historically been targeted during poultry production as a pathogen for reduction. Antunes et al., (2016) suggested loads of up of 10^6 to 10^8 of *Salmonella* are required for healthy adults to exhibit symptoms of illness. However, much lower concentrations of the pathogen may result in infection of children, or adults with compromised immune systems. The intensity of *Salmonella* spp. infection in humans depends upon the general health and immunity of the host, and as such, it becomes difficult to predict toxicity levels across populations.

Ingestion of pathogenic strains of *Salmonella* can result in any of a group of gastrointestinal issues, the severity of which may range from asymptomatic to life threatening. Most illnesses result in symptoms at 12 to 72 hours after consumption of the pathogen (CDC, 2018), which either resolve on their own or may result in hospitalization. A few cases may result in a form of arthritis, which may last for years.

Salmonella, like *Campylobacter*, prefer warm, damp environs and can thrive in processing environments. *Salmonella* levels may be influenced by variables other than just exposure, such as the age and stress level of the birds, the serotype and the genetic composition

of the host (Ricke et al., 2015). As *Salmonella* are looking for food, the gastrointestinal tract of birds is an opportune environment (Ricke et al., 2015; Rohmer et al., 2011).

Salmonella have historically been problematic to remove during poultry processing. For one, bacteria concentrations are highly variable and their reaction to cidal agents may not always be similar (Rodriguez et al., 2016). Adding to the difficulty of eradicating *Salmonella* is that the cells can congregate and form a biolfilm on processing equipment. Chemical interventions must be consistently monitored to ensure adequate concentration or the bacterial load can quickly increase (Corradini and Peleg, 2006).

Physical and chemical interventions have been utilized in poultry processing facilities to remove both debris and bacteria from bird carcasses. At almost any juncture in the plant environment an intervention may be applied to meet Agency or internal pathogen standards. The type and amount of intervention applied may be dependent upon efficacy, allowable limits, and financial considerations. Choices may also be made with respect to the chemical makeup of the facility's groundwater and/or organic load coming in from the grow-out facilities.

Data were collected as part of a process pathway analysis and, as such, intervention type was limited to what was implemented at participating facilities. Peracetic acid (PAA) is a frequently utilized intervention in both first and second processing, so it is heavily weighted in the analysis. PAA is composed of acetic acid and hydrogen peroxide, which makes it a safe choice for both sprays and dip applications, both on equipment and on poultry carcasses. Its use is considered less environmentally harmful, and more oxidizing, than chlorine (USDA, 2018).

Results indicate that PAA can be more effective than chlorine at reducing both *Salmonella* and *Campylobacter* (Nagel et al., 2013), which may be the result of PAA being more successful at negating an increased pathogen load (Brinez et al., 2006).

Chlorine is a much less expensive alternative than PAA, but can lose effectiveness in the presence of high organic loads (Buncic and Sofos, 2012) and reduce product quality if its concentration is too high. Chlorine tends to be most efficacious when there is an opportunity for continuous exposure to product, such as in an immersion chiller (McKee, 2012), although some studies have shown that it is not as effective in the chiller as in other locations (Buhr et al, 2005). Despite the usefulness of chlorine, its presence on poultry product is banned in several countries, thus making application an impossibility if product is destined for international sale. FreshFX®; a mixture of citric, phosphoric, or sulfuric acids can reduce pathogen loads in spray cabinets and has been used with success during processing. Cecure®, trade name for cetylpyridium chloride (CPC) was approved for use in 2004 for prechill spray cabinet or dip applications (Gilbert et al., 2015; Heiberg, 2004) and although not as common in use as PAA or chlorine, is still being utilized at processing facilities.

The objective of this study will focus on the influence of line speed and presence of a brush cabinet on *Campylobacter* and *Salmonella* reductions at the postpick location. Additionally, the reduction of these pathogens at the prechill, main chill, and postchill locations will be assessed as the result of recorded combinations of intervention concentration and pH. Results will be useful as an addition to the current literature on the efficacy of these interventions under specific conditions and locations.

4.3 Materials and Methods

Data were separated for each of the following locations (nodes): prescald, postpick, prechill, directly after the on-line reprocessing (OLR) rinse, postchill, frames (at the end of the debone line), drums and breast product (directly after the parts dip tank), and pre-grind product (whole leg or trim) directly before the grinder. These nodes were included in the model based upon the designated process flow (parts, ground, or MSC product).Samples from five poultry processing facilities were collected and recovered for *Campylobacter* spp. and *Salmonella* spp. over three seasonal cohorts (spring/fall, summer, and winter). Five samples each were taken at each of the locations during each sampling event (day) and reductions were taken by subtracting each individual value for a location from the mean of all samples from the prior location. This provided individual values of reduction while maintaining the within-flock data flow.

Values were $\log_{10}$ transformed before evaluation. Before transformation, values of less than the LOD were given the value of 1 log less than that value (e.g., <10 would be transformed to 1). Conversely, values that were recorded as greater then a number were given the value of 1 log greater than that number (e.g., >250 would be transformed to 2500). For *Campylobacter*, values of 0 were imputed to 0.1, and for *Salmonella* <0.03 was changed to 0.02 as the $\log_{10}$ transformation of 0 is indeterminable.

Whole bird carcass rinse (WBCR) samples were sent to a corporate laboratory and stored (from 2 hours to overnight) at a temperature range of 35.6 to 39.2ºF. The *Salmonella* samples were prepared by aseptically transferring 20 ml of rinsate and 30 ml of fresh buffered peptone water (BPW) into a sterile specimen cup for each of the samples. All bags were labeled by batch and controls were prepared and all were incubated for determination.

Presence/absence tests were conducted with polymerase chain reaction (PCR) screening using the DuPont Qualicon BAX® method. *Salmonella* spp. samples with positive results were directly tubed and enumerated with most probable number (MPN) analysis and *Campylobacter* spp. was enumerated with chromogenic agar plates (specific for *Campylobacter jejuni* and *Campylobacter coli*). *Campylobacter* rinses were set with a 1:1 dilution, with .50 ml each of dilution spread across 2 agar plates. The inoculum was allowed to dry completely before incubation and dishes were examined at 48 +/- 2 hours, with the sum of both plates being reported.

Results from first processing were chosen for analysis as previous sensitivity analyses indicated that pathogen loads at process end were most heavily affected by reductions that occurred up to the end of the immersion chiller (Chapter 3). For each of three locations, post-pick, prechill (after the OLR cabinet), and postchill, reductions were collected for the attributes listed in Table 4.1.

An Analysis of Variance (Proc GLM) in SAS (Statistical Analysis Software) was conducted to determine if there was a significant difference between the mean $\log_{10}$ counts for line speed and cabinet type (post-pick location) and intervention type (prechill and postchill locations). Plant location was included so that these differences were accounted for in the model. The analysis was designed with the following formula:

$$R_{ijk} = \mu + C_i + L_j + T_k + LC_l + \epsilon_{ijkl} \text{ where;} \quad (4.1)$$

R_{ijk} is μ, the overall mean; C_i is the effect of the cabinet type, (cabinet or no cabinet); L_j is the effect of the j^{th} line speed (105 or 140); T_k is the effect of the k^{th} treatment (PAA, chlorine, mixed

acid, or Cecure®); LC_l is the effect of the l^{th} sampling location, and ϵ_{ijkl} is the model error. Due to sample size, interactions were not introduced into the model.

Table 4.1 Factors analyzed by location and attributes.

Location	Factor	Attribute
Postpick	Line speed	105 bpm 140 bpm
	Post-pick steam cabinet	cabinet no cabinet
Prechill	Intervention	PAA FreshFX®
	pH	continuous
	ppm	continuous
Postchill	Intervention (main chill)	chlorine PAA
	Intervention (Finishing chill)	FreshFX® PAA Cecure®
	pH (Main chill) pH (Finishing chill)	continuous
	ppm (Main chill) ppm (Finishing chill)	continuous

Models were analyzed using Multiple regression analyses (Proc REG) models for each intervention type and location combination to determine if pH and ppm levels could be used as predictors for pathogen levels at output. The linear model was fit as follows:

$$y_i = \beta_0 + \beta_{1x1} + \beta_{2x2} + \epsilon_{x1,x2....xn}, \text{ where:} \quad (4.2)$$

β_0 is the intercept; β_{1x1} is the pH of the intervention; β_{2x2} is the concentration (ppm), and $\epsilon_{x1,x2....xn}$ is the error for each factor. A stepwise regression model analysis was used to remove factors from the model if needed, in which case only a single regression model was fit.

4.4 Results

4.4.1 *Postpick reductions*

Mean $\log_{10}$ *Campylobacter* spp. reductions at the postpick location were not significantly different by line speed category, but there was a significant difference between *Salmonella* spp. reductions, with slower line speeds resulting in larger decreases (Table 4.2). However, the numerical log difference is actually quite small (.03 $\log_{10}$), indicating that the presence of a postpick steam cabinet resulted in a significantly higher reduction of *Campylobacter* spp. Conversely, *Salmonella* spp. exhibited a higher decrease at post-pick at facilities without cabinet addition.

4.4.2 Prechill reductions

At the prechill location, the PAA intervention resulted in a higher *Campylobacter* spp. reduction from the previous location than the product treated with the FreshFX®. However, FreshFX® was more successful in reducing *Salmonella* spp. load than was PAA in the on-line reprocessing (OLR) station (Table 4.3).

The slope for the prechill *Salmonella* spp. reduction model was positive and significant for FreshFX® for both *Campylobacter* spp. and *Salmonella* spp. and for PAA for *Salmonella* spp. There was not a statistically significant relationship between the pH of the PAA application and *Campylobacter* spp. reduction. For the FreshFX® intervention, *Salmonella* spp. reduction was significant for pH (ppm is not collected to monitor this intervention), although the model fit was somewhat weak (R^2= 0.21). This was also the case for the *Campylobacter* spp. reduction as a result of the pH influence the FreshFX® application, which was significant, but quite weak as

Table 4.2 Mean Log_{10} reductions by line speed and cabinet use at the postpick location

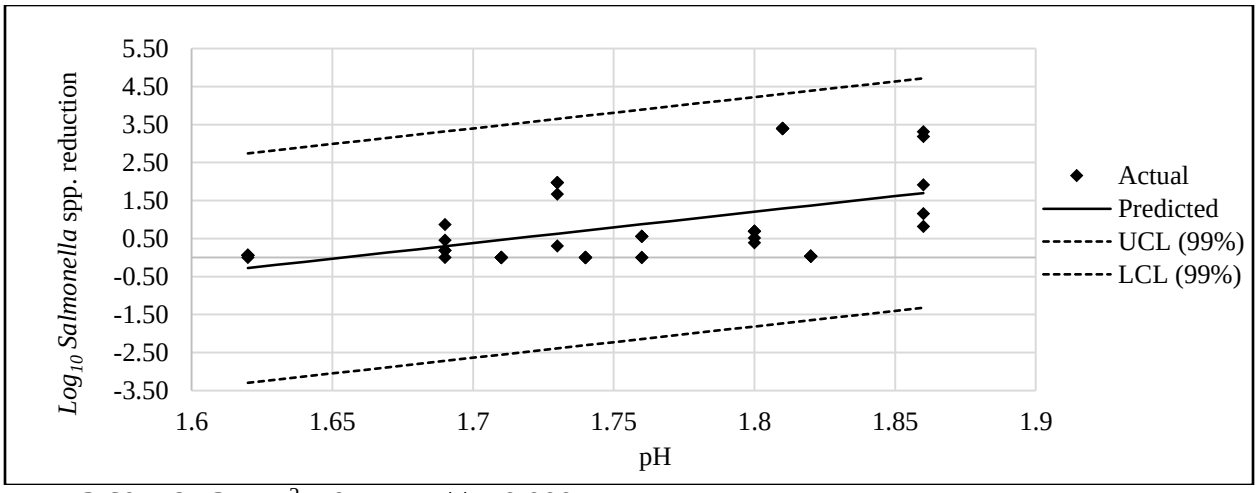
Pathogen type	BPM 105	BPM 140
Mean log_{10} *Campylobacter* spp. reduction	2.00[a]	2.11[a]
Mean log_{10} *Salmonella* spp. reduction	1.63[a]	1.60[b]
	Cabinet	**No Cabinet**
Mean log_{10} *Campylobacter* spp. reduction	2.17[a]	1.94[b]
Mean log_{10} *Salmonella* spp. reduction	1.35[b]	1.88[a]

[ab] Differing superscripts within each row were significantly different ($p \leq 0.05$).

Table 4.3 Mean Log_{10} reductions by prechill treatment

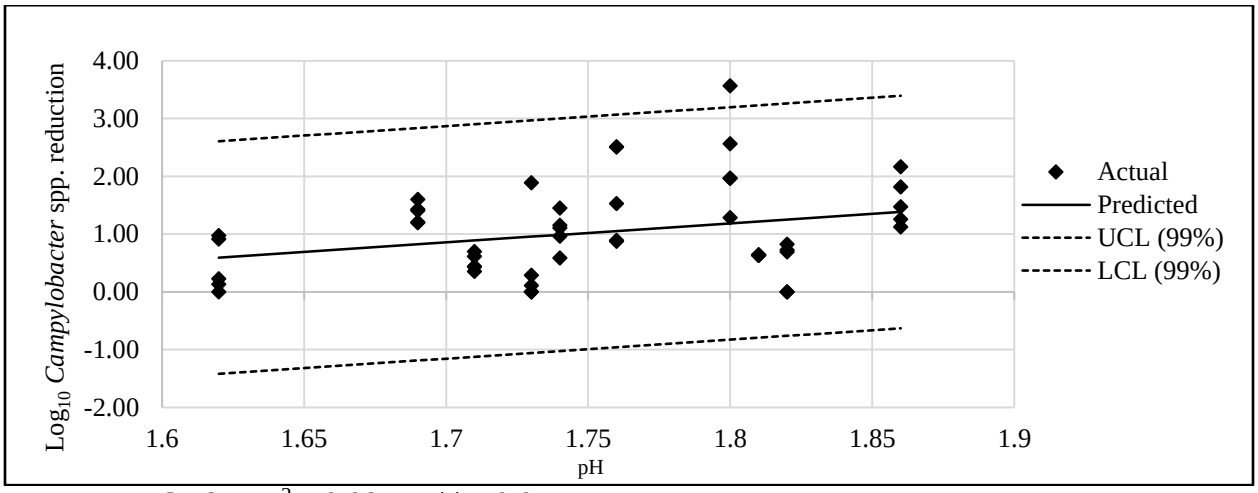
Pathogen type	PAA	FreshFX®
Mean log_{10} *Campylobacter* spp. reduction	1.54[a]	1.04[b]
Mean log_{10} *Salmonella* spp. reduction	0.27[b]	0.84[a]

[ab] Differing superscripts within each row were significantly different ($p \leq 0.05$).



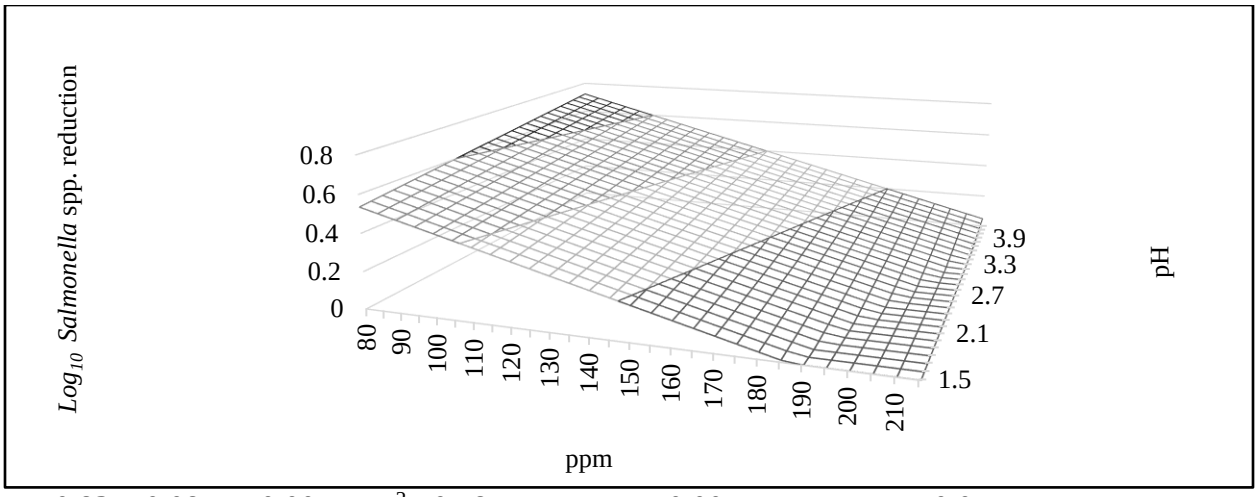
$y = -13.60 + 8.23x$, $R^2 = 0.21$ $P > |t| = 0.0004$

Figure 4.1 *Salmonella* spp. reduction for FreshFX® application at prechill



$y = -4.72 + 3.28x$, $R^2 = 0.08$ $P > |t| = 0.042$

Figure 4.2 *Campylobacter* spp. reduction for FreshFX® application at prechill



$y = 0.83 + 0.08x_1 – 0.005x_2$, R^2= 0.28 ppm Pr > F =0.0014, pH Pr > F = 0.014

Figure 4.3 *Salmonella* spp. reduction for PAA application at prechill

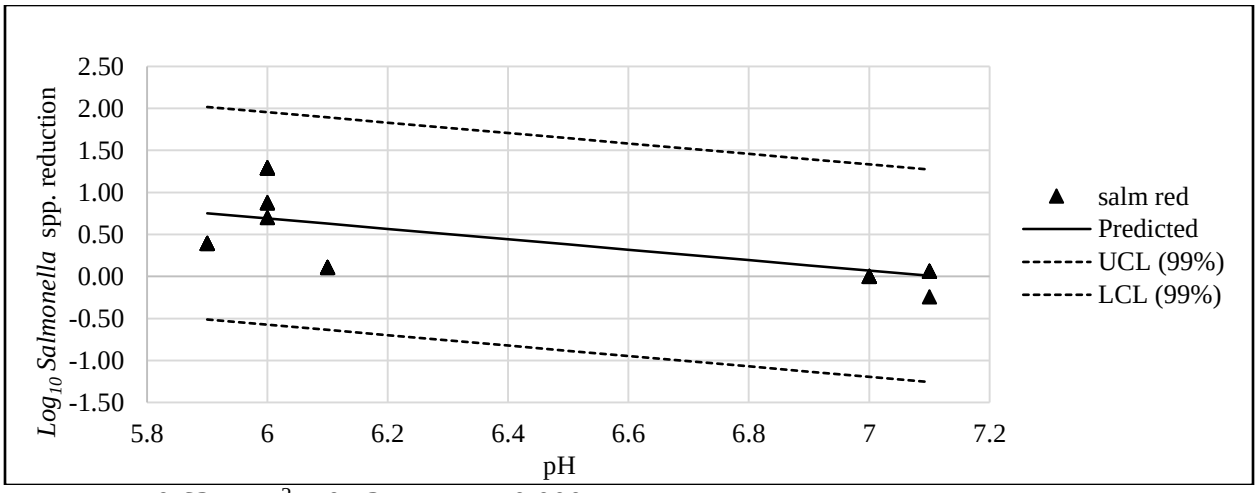
well (R^2= 0.08). For the PAA intervention, the stepwise regression analysis kept both pH and ppm in the model with a lower strength relationship (R^2 =0.28).

Results of the reductions indicated that for the FreshFX® intervention an increase in the pH resulted in an increase in both *Salmonella* spp. and *Campylobacter* spp. reduction (Figures 4.1 and 4.2), an increase in the pH resulted in an increase in both *Salmonella* spp. and

Table 4.4 Mean log_{10} reductions by factor type at the main chiller

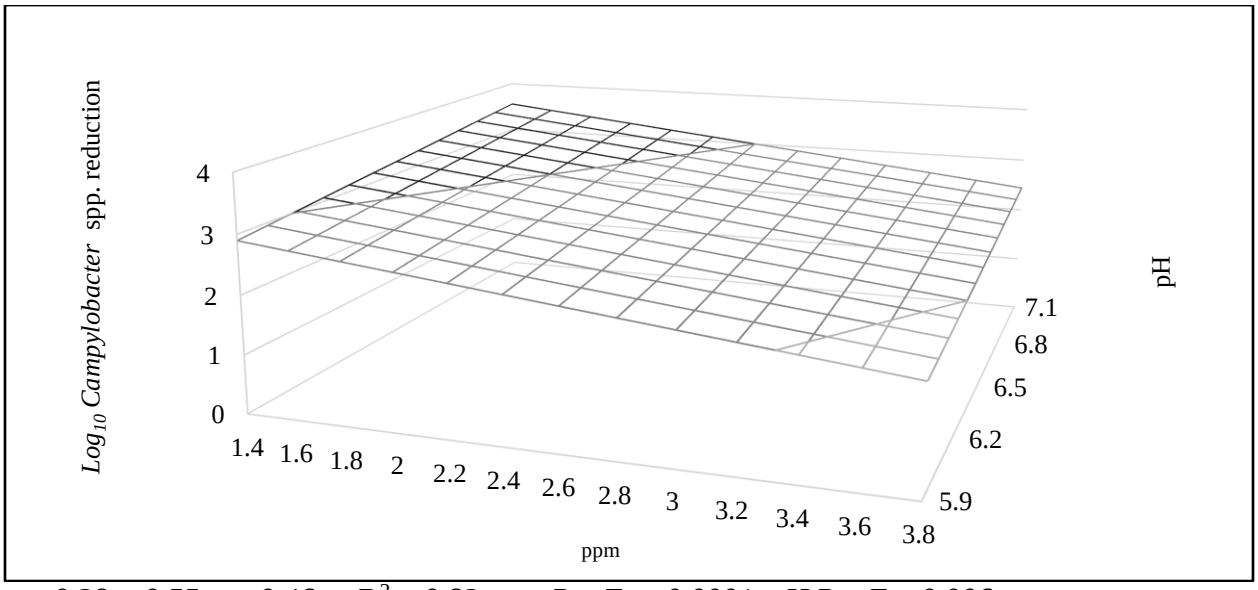
Pathogen type	Chlorine	PAA
Mean log_{10} *Campylobacter* spp. reduction	2.64[a]	1.83[b]
Mean log_{10} *Salmonella* spp. reduction	0.43[a]	0.19[b]

[ab] Differing superscripts within each row were significantly different ($p \leq 0.05$).



$y = 4.41 + -0.63x_1$, $R^2 = 0.43$. Pr > F = 0.0001

Figure 4.4 *Salmonella* spp. reduction using chlorine at the main chiller



$y = 0.29 + 0.55x_1 – 0.46x_2$, $R^2 = 0.82$ ppm P > F= <0.0001, pH P > F = 0.006

Figure 4.5 *Campylobacter* spp. reduction using chlorine at the main chiller

4.1 and 4.2). For the PAA intervention at the prechill location, the log_{10} *Salmonella* spp.

Campylobacter spp. reduction. For the PAA intervention at the prechill location, the log_{10} *Salmonella* spp. reduction was highest at the intersection of lower concentration of PAA and high levels of pH (Figure 4.3).

4.4.3 *Main chiller reductions*

Pathogen reductions at the exit of the main chiller were significantly higher for the chlorine intervention than for the use of PAA in the chill water (Table 4.4). Linear estimates of the reduction were also significant for the chlorine intervention (Figures 4.4 and 4.5), with pH being retained in the stepwise analysis for the *Salmonella* spp. model and both pH and chlorine concentration in the *Campylobacter* spp. model. Neither of the PAA intervention models at the main chiller water were significant for the reduction of either *Salmonella* spp. or *Campylobacter* spp. and were, therefore, not included in the model.

Regression results indicated that for chlorine in the main chiller, *Salmonella* reductions decreased as pH levels increased (Figure 4.4). Conversely, *Campylobacter* spp. reductions increased as the result of higher pH and lower chlorine concentration (Figure 4.5). The relationship between *Salmonella* and pH was moderately strong (R^2= 0.43), whereas the weight of the relationship between *Campylobacter* reduction and pH and chlorine concentration is much more robust (R^2= 0.82)

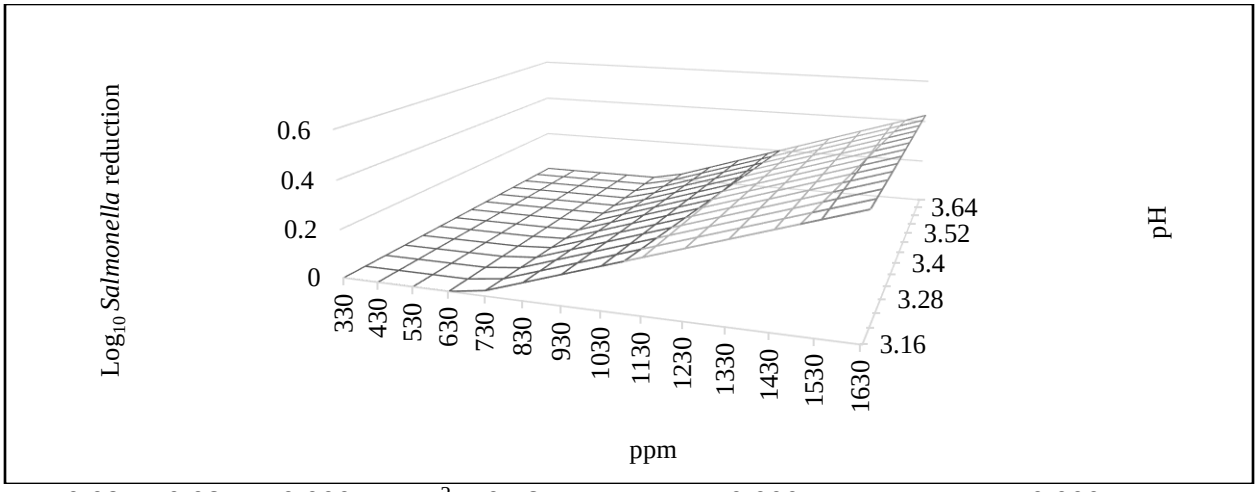
4.4.4 *Finishing chill reductions*

At the finishing chiller, the highest reduction of *Campylobacter* spp. was the result of application of FreshFX®. There was not a statistically significant difference for the *Salmonella* spp. reduction between the use of FreshFX® and Cecure® (Table 4.5). However, both reductions were higher than that for PAA at the finishing chiller, the use of which resulted in the lowest cidal activity for both pathogens.

Table 4.5 Mean log_{10} reductions by factor type at finishing chiller

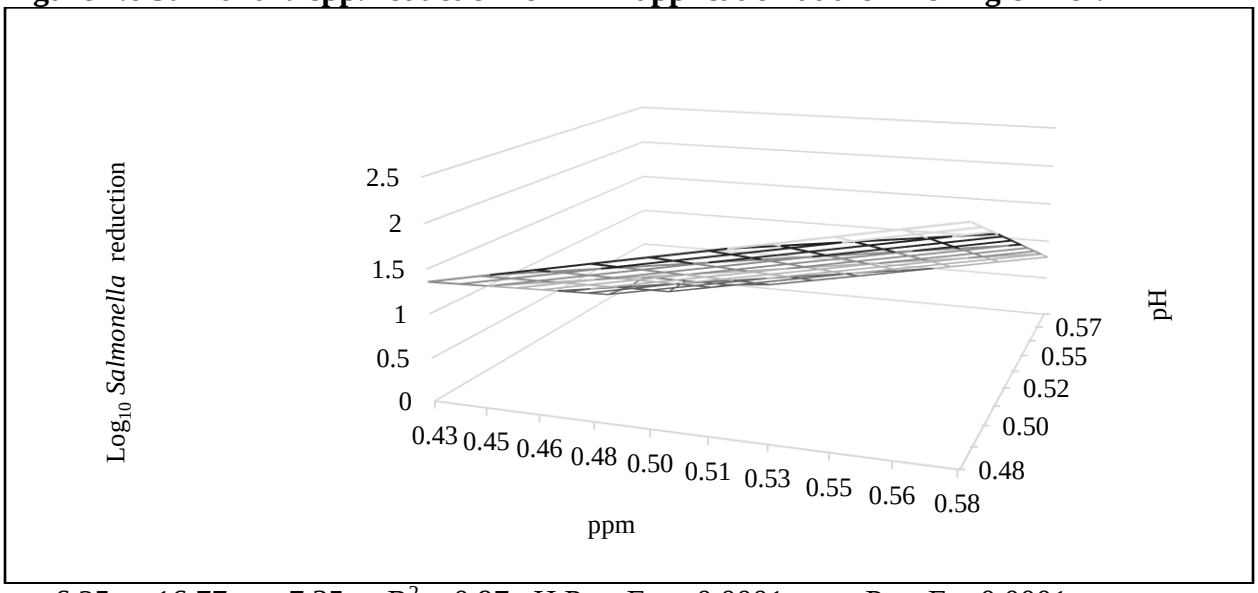
Pathogen Type	FreshFX®	PAA	Cecure®
Mean log_{10} *Campylobacter* spp. reduction	2.67[a]	1.83[b]	1.59[b]
Mean log_{10} *Salmonella* spp. reduction	0.46[a]	0.01[b]	0.57[a]

[ab] Differing superscripts within each row were significantly different ($p \leq 0.05$).



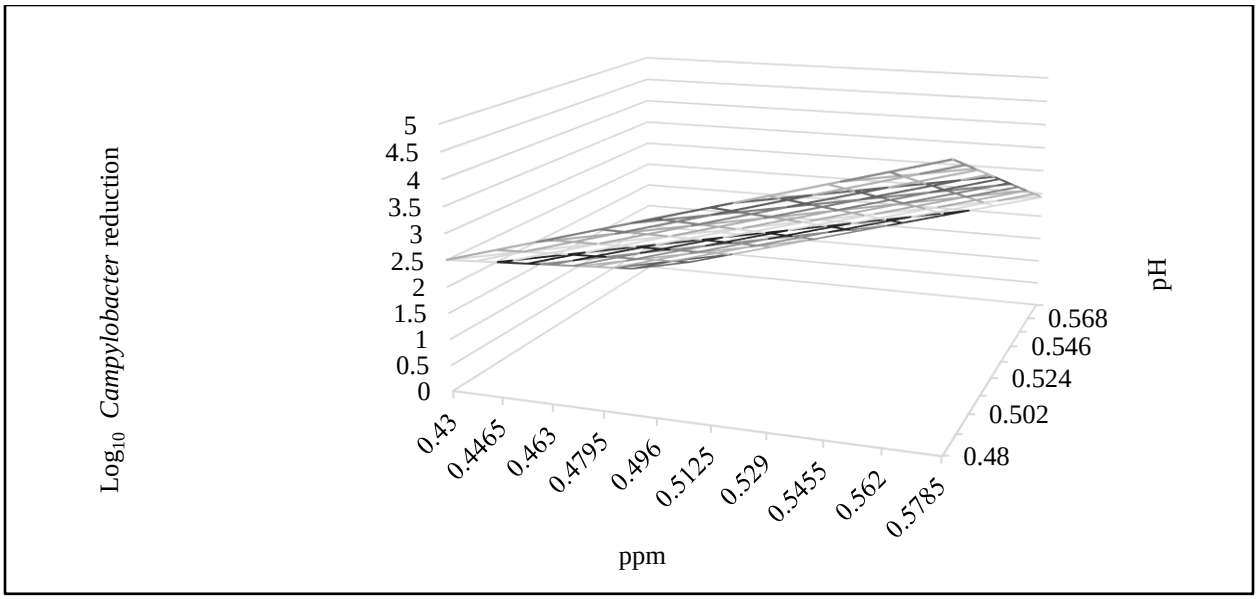
$y = -0.09 + -0.08x_1 + 0.0005x_2$, $R^2 = 0.78$ pH Pr > F = <0.0001, ppm Pr > F = <0.0001

Figure 4.6 *Salmonella* spp. reduction for PAA application at the finishing chiller.



$y = 6.25 + -16.77x_1 + 7.35x_2$, $R^2 = 0.97$ pH Pr > F = <0.0001, ppm Pr > F = 0.0001

Figure 4.7 *Salmonella* spp. reduction for Cecure® application at the finishing chiller



$y = -25.99x_1 + 16.75x_2 + 7.78$, $R^2 = 0.68$ pH Pr > F = <0.0001, ppm Pr > F = 0.0001

Figure 4.8 *Campylobacter* spp. reduction for Cecure® application at the finishing chiller

At the finishing chiller, the relationship between *Salmonella* spp. and *Campylobacter* spp. reduction, pH level, and FreshFX® concentration was not significant (Figures 4.7 and 4.8). For the PAA intervention, *Salmonella* spp. reduction was highest at lower pH and a stronger concentration. (There was not a significant relationship for the PAA treatment and *Campylobacter* pH and concentration.) The Cecure® intervention was most effective against *Salmonella* spp. and *Campylobacter* spp. when the pH was low and concentration was high.

4.5. Conclusion

This study covers the effect of line speed and cabinet (presence or absence) at postpick, and intervention type, pH, and concentration at the prechill, main chiller, and finishing chiller locations. The application of physical and chemical interventions at different locations with processing plant resulted in reduction of *Salmonella* and *Campylobacter* counts with some interventions having significantly greater reduction potential than others. Although public opinion is that line speed may affect the safety of food, the data examined did not exhibit a

significant difference between line speeds for the *Campylobacter* spp. mean reduction. The *Salmonella* spp. mean reduction was significantly higher statistically for the lower line speed, but the difference was so small (.03 $\log_{10}$) that it may not be biologically significant.

Introduction of a post-pick steam cabinet to the pre-evisceration process did result in a significantly higher *Campylobacter* spp. reduction at the post-pick location than for those facilities that did not have a steam cabinet. This situation was reversed for the pattern of *Salmonella* spp. reduction where the presence of a steam cabinet resulted in a lower mean decrease than without. There is no indication as to why the steam cabinet would be more effective at *Campylobacter* spp. reduction than for *Salmonella* spp. It is possible that the fragile nature of the *Campylobacter* bacterium (Silva et al., 2011) was more affected by the heat of the steam cabinet than that of *Salmonella*.

Salmonella spp. exhibited a higher reduction for PAA than for the mixed acid at the prechill location, whereas *Campylobacter* spp. had a larger reduction with the FreshFX® intervention. Reduction in *Salmonella* spp. and *Campylobacter* spp. from the post-pick to the prechill location (directly out of the OLR) increased as the pH increased for the mixed acid applications (FreshFX®). For the PAA intervention at the same location, reductions were highest at the junction of higher pH (3.1 to 4.2) and lower concentration (80-95 ppm) where *Salmonella* spp. reductions were between ~ 0.62 to 0.72 $\log_{10}$. There was not a statistically significant relationship between either pH or ppm for the *Campylobacter* spp. reduction model.

Use of the chlorine intervention in the main chiller resulted in a significantly higher reduction for both *Salmonella* spp. and *Campylobacter* spp. than for PAA. A linear model for the chlorine intervention showed the highest *Campylobacter* spp. reductions when the pH was high (6.8 to 7.1) and the concentration was lower (1.4 to 1.8), resulting in decreases of 3.4 to 3.6 $\log_{10}$.

Conversely, for *Salmonella* spp., chlorine at the main chiller resulted in a higher reduction when pH decreased, with a 0.75 $\log_{10}$ decrease when the pH was lowered to 5.9. Neither pH nor concentration was significantly related to pathogen reduction for both *Campylobacter* and *Salmonella*.

FreshFX® applied in the finishing chiller resulted in the highest reduction for *Campylobacter* spp. postchill, with reductions resultant from PAA and Cecure® application not being significantly different from one another ($P > 0.05$). For *Salmonella* spp. in the finishing chiller, FreshFX® and Cecure® application exhibited reductions that were significantly higher than those for the PAA intervention. Neither of the pH or concentration were included in a model for the FreshFX® application for reduction of either pathogen or for the *Campylobacter* spp. reduction for PAA use.

Salmonella spp. reduction as a result of the PAA intervention in the finishing chiller was highest when the pH was low (3.16 to 3.40) and the concentration was high (1530 to 1630 ppm), resulting in reductions from 0.45 to 0.47 $\log_{10}$. For the use of Cecure® in the finishing chiller, both pathogen reductions were increased by maintaining a low pH (0.48 to 0.49) along with a higher concentration (0.56-0.58 ppm), resulting in a possible 4.5 to 4.99 $\log_{10}$ reduction for *Campylobacter* spp. and approaching a 2.20 to 2.44 $\log_{10}$ reduction for *Salmonella* spp.

4.5.1 Sources of error

Even though the results of this analysis are of note, the sample sizes were small for each factor level. A power analysis assuming a 0.75 $\log_{10}$ (biologically significant) difference between groups, a standard deviation of 1.00 (a level of variation commonly found in processing facility samples), and the traditionally accepted power of 0.80 would require a sample size of 58.

However, the experimental design for this study was limited both by time and cost factors with sample sizes varying by location and intervention type from 20 to 50. In addition, the factor types were delineated along the same lines as specific processing facilities, which may have introduced multiple confounding variables to the analyses. The significant cost of sampling and collection made larger sample collections infeasible so following flock through the process was the most logical decision for these models.

The difference from one location to the next in the process was calculated by subtracting each individual bird rinse (n = 5), randomly selected, from the mean of bird rinses (n = 5) from the previous node, which were also randomly selected. Although this method had limitations, it enabled the use of the samples from the location of interest as individual values, resulting in larger sample sets than if mean differences were calculated. It also enabled a collection scheme that was much more physically reasonable than attempting to follow the same birds throughout the process. However, this may have resulted in less variation between reductions as may have been found if the samples had been collected from the same birds.

Due to the very low levels of pathogens in the processing environment, it was necessary to impute high frequencies of <1 for *Campylobacter* spp. and <0.03 for *Salmonella* spp. These high frequencies of low values resulted in very little variation in y (reduction) within each location and may have shifted the actual mean of the analysis. In addition, in a few cases (as these were not the same birds from one location to another) there were increases in individual rinses at a location. These values were adjusted to a reduction of 0, which may have aided in model fit, but skewed the interpretation.

The use of buffered peptone water (BPW) to rinse the bird carcasses may have resulted in lower counts, specifically for PAA in the rinsate. Recent studies have shown that neutralized BPW (nBPW) may be more efficient in estimating existing bacterial counts in bird rinses where PAA has been utilized as an intervention (Taylor and Vuia-Riser, 2017). In this case, nBPW was not available so carcasses were drained for approximately1 minute before rinsing. It is possible that using nBPW would have resulted in more recovery and, thus, more variability between samples.

4.5.2 Suggestions for further research

The experimental methodology used for the reduction analysis could be more beneficial when applied to a much larger sampling design. Specifically, more processing facilities and more samples at each location should be represented. In this study, the results were assumed to be generalized for the entire industry as there were not enough samples by location for each facility cohort, and, the models could not be blocked by plant. Cost restrictions will make it necessary for a study of this nature to accommodate data from several researchers over time, but following individual flocks through a process is integral to understanding the intersectional role of intervention type, pH, and concentration in pathogen reduction.

The use of nBPW may prove necessary to a more representative recovery of pathogens, at least when PAA is the intervention used at a location. As more research into the use of nBPW on carcass rinses becomes available a relationship between the two methodologies may be further defined, making predictions about recovery error with BPW possible.

A controlled study with inoculated product would be one way to approach the best combination of factors while starting with enough microbial load to understand how these

reductions actually work. However, valuable information from a mechanistic process may be lost when the study is taken outside of the processing environment. One way to incorporate the processing environment along with dealing with multiple zeros found naturally would be to define a relationship between a pathogen of interest and an indicator organism. Models for poultry pathogen reduction will continue to be refined for benefit of the industry.

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Chapter 5 Consideration of *Enterobacteriaceae* as an Indicator Organism for *Campylobacter* spp. and *Salmonella* spp. in Poultry Processing

5.1 Abstract

The use of indicator organisms as a gauge for food safety and sanitation has been employed in the poultry industry for over twenty years (Mead, 2007, Roccato et. al., 2018; Schaffner and Smith-Simpson, 2014; Williams et al., 2017; Zeitoun et al., 1994). *Enterobacteriaceae* has been traditionally used as an indicator of process sanitation, with less emphasis given to the possibility of utility as an indicator of pathogen activity in meat processing, with Escherichia coli more often employed for this purpose (Dehalle et al, 2009; Wages et al., 2014; Williams et al., 2017). However, some studies have indicated that the *Enterobacteriaceae* group contains more microorganisms of concern to food safety (Mossel and Struijk, 1995).

Samples taken directly from a mechanized poultry process were followed through first and second processing, with each sample being processed for recovery of *Enterobacteriaceae, Salmonella* spp., and *Campylobacter* spp. The sampling design included samples from different processing facilities and flocks in order to adequately represent multiple processes. Counts were analyzed with a generalized linear model analysis by location/part type for *Enterobacteriaceae* by *Salmonella* spp. and *Campylobacter* spp.

Results revealed that there was a significant relationship between *Enterobacteriaceae* and *Salmonella* spp. for pre-scald and post-chill whole bird carcass rinses (WBCR's), and for parts (consisting of drums, breast, and whole leg samples). *Enterobacteriaceae* and *Campylobacter* spp. counts were also significantly correlated for the pre-scald and pre-chill location WBCR's. However, for all these results, the correlation coefficients were low (less than 0.50), suggesting variation for the pathogens of interest was not highly affected by the level of *Enterobacteriaceae*

in the product. Future studies will require more focused sampling at each location throughout the process.

5.2 Introduction

Internationally, reported infections from foodborne Salmonellosis are responsible for approximately 80 million reported cases of illness each year (Hansen and Malorny, 2016). *Salmonella enteritidis* and *Salmonella typhimurium* are the serovars most highly linked to human illness from consumption of poultry product. The infective dose of the pathogen is a matter of opinion, with most studies suggesting a range between 10^3 to 10^4 organisms (Hansen and Malorny, 2016), although virulence of these bacteria may be more complicated than previously considered, depending on multiple factors not entirely dependent on host susceptibility (Bumann and Cunrath, 2017).

Campylobacter spp. is the 2nd most common cause of bacterial illness in United States, behind *Salmonella* (CDC, 2018; Lourdes Garcia-Sanchez et al., 2018; Roccato et al., 2018), with poultry consumption being impugned with the majority of cases (EFSA, 2011). Of the genus *Campylobacter*, *C. jejuni* is of the most concern for poultry processors as it is most associated with illness via consumption (Newell et al., 2017; Gruntar et al., 2015).

Indicator organisms in meat and poultry are often used to make predictions about the behavior of pathogenic bacteria in a process (Habib et al., 2012; Mead 2007; Roccato et al., 2017; Schaffner and Smith-Simpson, 2014; Wages et al., 2014; Zeitoun et al., 2004). As foodborne pathogens are often found in extremely low concentrations in the processing environment (< 1 cfu/g), reduction studies may not be meaningful (Bollerslev et al., 2017). However, laboratory experiments (where product may be inoculated with the micro pathogen)

cannot adequately represent the variability encountered in processing situations. Using an organism that can be sampled as a substitute for a foodborne pathogen while still being found in large enough counts to determine a possible reduction is needed.

Mead (2007), and Schaffner and Smith (2004) listed some requirements for a useful indicator organism, such as, that the indicator must be recoverable when the pathogen of interest is found in a product or process. Even so, the indicator microbe must also be commonly found so that a reduction of the same can be measurable between locations or interventions. Naturally, the two organisms must have a strong relationship with one another so that reduction (or increase) in one will be mirrored in the other. Last, it is beneficial that the indicator organism is easy to recover in the lab, so that it is cheaper and easier to enumerate than the pathogen of interest.

Enterobacteriaceae is often used as an indicator of poor sanitation practices (Altekruse, et al., 2009; Biasino et al., 2018; Halkman and Halkman, 2014; Williams et al., 2014), but has also been considered as a gauge for fluctuation of pathogen levels (Guenther et al., 2010; Roccato et al., 2018). As part of a large system of gram-negative facultative anaerobes, including *Salmonella*, *Enterobacteriaceae* may be useful in representing pathogenic bacteria found in the gut of animals at slaughter. As the bacteria may be commonly found in poultry processing, and in larger numbers than either *Salmonella* spp., it supplies the recommended experimental design of following reductions in the process. It is certainly much easier to incubate and recover in the laboratory than *Salmonella*. There are no available studies where *Enterobacteriaceae* has been investigated as an indicator for *Campylobacter*.

Cibin et al., (2014), and later, Roccato et al., (2018) evaluated the efficacy of *Enterobacteriaceae* in determining both the presence and count of *Campylobacter* on poultry neck skin and found that increasing levels of *Enterobacteriaceae* corresponded with increasing

levels of *Campylobacter* at 2 points in the system (post-evisceration and post-chill). However, Belluco et al., (2015) did not find a significant change in *Enterobacteriaceae* counts after post-pick and washing (because this was an EU study so the samples were air-chilled rather than processed through an immersion system).

A study of the relationship between *Enterobacteriaceae* and *Salmonella* presence in a pig slaughter processes (Biasino et al, 2018; Delhalle et al., 2008) found a positive correlation between *Enterobacteriaceae* counts and *Salmonella* presence on certain portions of pork carcasses. Handley et al., (2016) suggests that *Enterobacteriaceae* may be a useful indicator of the *Salmonella* cells that manage to burrow inside the skin of poultry carcasses.

In this study, *Enterobacteriaceae* counts on raw poultry product in first and second processing are compared to *Salmonella* spp. and *Campylobacter* spp. counts and analyzed to determine if process increases are significantly correlated to one another. Chilled and stored and frozen product were not considered for this analysis as previous work has indicated that a psychotropic species of *Enterobacteriaceae* can grow in this environment and is not a good predictor of either *Salmonella* or *Campylobacter* in poultry product (Zeitoun et al., 1994).

5.3 Materials and Methods

5.3.1. Sampling Methods

Poultry product samples from 5 separate production facilities and 22 flocks were collected over a period of 7 months (January to July, 2016). For each flock, 5 samples were taken from several locations (prescald, postpick, prechill, postchill, frames, mechanically separated chicken product (MSC), ground product, and parts product), with the overall pathway differing by sampling event.

For the prescald, postpick, prechill, and postchill samples, whole bird carcass rinses (WBCR's) were collected by randomly selecting and aseptically collecting a poultry carcass off the line and rinsing in 400 ml of cooled buffered peptone water in an arcing motion for a period of approximately 1 minute. For the frame samples, one frame was randomly selected at the end of debone and rinsed in 200 ml of buffered peptone water, while the MSC and ground samples were collected in approximately1 lb. samples and sent to the laboratory for stomaching before analysis. Parts product consisted of approximately 4 lbs. of either breast, drums, or whole leg product rinsed in 400 ml of buffered peptone water.

5.3.2 Recovery Methodology

Samples were sent to the corporate laboratory and stored at a temperature range of 35.6 to 39.2º F. For *Enterobacteriaceae* recovery, samples were allowed to come to room temperature and 1 ml of diluted samples was directly applied to 3M® *Enterobacteriaceae* petrifilm and incubated at 35 ± 1º C for 22-26 hours, after which time colonies were counted and recorded.

Salmonella spp. samples were first analyzed for presence/absence with polymerase chain reaction (PCR) screening using DuPont Qualicon BAX® methodology. Positive MSC and ground sampless were then tested with most probable number (MPN) analysis by diluting 65 g of sample in 585 ml of enrichment broth in a 1:10 dilution and stomached for 2 minutes. These samples, along with the rinse samples were added in 10 ml increments to 3 tubes, which were then further diluted to create three 1 ml tubes and again in three 0.1 ml tubes. Table references were used to determine counts according to BAM (Bacteriological Analytical Manual) procedures (Blodgett, 2010). *Campylobacter* rinses were diluted 1:1, and each dilution was swabbed across chromogenic agar plates (specific for *Campylobacter jejuni* and *Campylobacter*

coli). At 48 ± 2 hours, with the samples having dried, plates were enumerated and counts recorded.

5.3.3. Data analysis

Counts were $\log_{10}$ transformed in order to prepare data for linear regression analysis. As the $\log_{10}$ transformation of 0 is undefined, *Enterobacteriaceae* and *Campylobacter* spp. 0 cfu/ml results were imputed to 0.1 before transformation. *Salmonella* spp. positives that were recorded as < 0.03 cfu/ml were transformed to 0.03 and negatives were changed to 0.02. Values that were recorded as greater than a given value were given a value of 1 log greater than that value. Estimated values were given the reported value.

Although data were collected by flock (which ran on the same line as processing facility), there was not enough data sampled at each opportunity for the model to be analyzed in this manner. An analysis of variance was evaluated by location in the process (Table 5.1) in order to delineate the changes in counts by pathogen and location type. Linear regression analyses were run by location for *Salmonella* spp. and *Campylobacter* spp. by *Enterobacteriaceae* to determine if there was a significant relationship between the indicator and pathogenic organisms.

5.4 Results

Descriptive statistics for all three microbial categories (Figure 5.1) by location indicate the highest counts were found at the pre-scald location, which is not surprising, as no interventions had yet been placed on the product. *Enterobacteriaceae* counts appear to go down throughout first processing in tandem with *Campylobacter* spp. counts whereas *Salmonella* spp. counts appear to slightly flatten at the post-pick location. Variation for all three groups was numerically highest in the MSC product, which is the result of multiple 0 results along with a

Table 5.1 Mean Log_{10} microbial values by location

		Log_{10} EB		**Log_{10} *Campylobacter* spp.**		**Log_{10} *Salmonella* spp.**	
Location/Type	N	Mean	SD	Mean	SD	Mean	SD
Pre-scald	110	4.99^{ax}	0.46	4.32^{ay}	1.25	0.57^{az}	1.50
Post-pick	110	2.89^{cx}	0.64	2.27^{by}	1.53	-1.15^{bz}	1.03
Pre-chill	111	1.79^{dx}	0.55	1.06^{cy}	1.24	-1.44^{cz}	0.60
Post-chill	112	-0.42^{fx}	0.87	-0.97^{ey}	0.20	-1.69^{cz}	0.04
Frames	64	1.05^{ex}	0.90	-0.90^{ey}	0.34	-1.56^{cz}	0.40
Parts	156	0.84^{ex}	1.11	-0.86^{ey}	0.61	-1.52^{cz}	0.47
Ground	35	1.85^{dx}	0.83	-0.86^{ey}	0.36	-1.42^{cz}	0.45
MSC	55	3.18^{bx}	0.55	-0.30^{dy}	1.10	0.38^{az}	1.27

^{abcdef} Differing superscripts by column indicate a significant difference by location for pathogen type ($p \leq 0.05$).
^{xyz} Differing superscripts by row indicate a significant difference by pathogen type for location ($p \leq 0.05$).

few higher counts. This higher variation has been suggested to increase risk (Duarte et al, 2015; Nauta et. al, 2005) when reduction at that location is low. Analysis of the counts within location by pathogen type show that *Enterobacteriaceae* counts were significantly higher than those for *Campylobacter* spp., which were higher than those for *Salmonella* spp. for every location.

A line chart of the means through first processing (Figure 5.1) show that as the log_{10} mean *Enterobacteriaceae* counts decrease the *Campylobacter* spp. mean log_{10} counts did as well. The *Salmonella* spp. mean log_{10} counts decreased along with the *Enterobacteriaceae* counts until the post-pick location, where it appears to flatten, trending slightly downward to postchill. *Enterobacteriaceae* counts were consistently higher than those for *Campylobacter*, which were consistently higher than those for *Salmonella*.

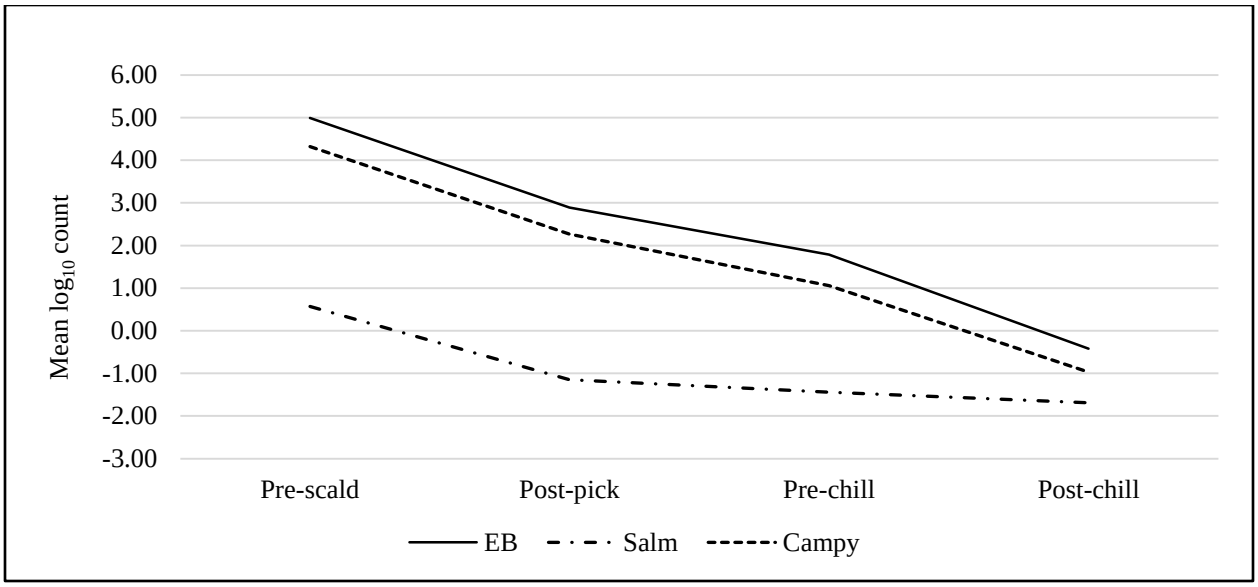


Figure 5.1 Mean $\log_{10}$ values by pathogen type for *Enterobacteriaceae*, *Salmonella* spp., and *Campylobacter* spp. for first processing WBCR's.

After first processing, the mean $\log_{10}$ *Enterobacteriaceae* counts are all positive (> 0) and are higher for the MSC and ground product than they are for the frames and parts product (Figure 5.2). However, the mean $\log_{10}$ *Salmonella* spp. and *Campylobacter* spp. counts were consistent for all part types (meaning that they were negative), with the exception of the MSC product, where both of the mean $\log_{10}$ values of these pathogens increased. This indicates that there is an aspect of the MSC process that is either resulting in an increase in pathogen counts, or the counts are more recoverable after this treatment.

As there were a preponderance of negative results for the *Salmonella* spp. and *Campylobacter* spp. samples, Tobit models were assessed for the first processing WBCR's and the other product types to determine if there was a relationship between *Enterobacteriaceae* and *Campylobacter* spp. and *Salmonella* spp. Proc Lifereg in SAS (statistical analysis software) was employed to create the models, which were used to define the relationship between these pathogens and *Enterobacteriaceae*.

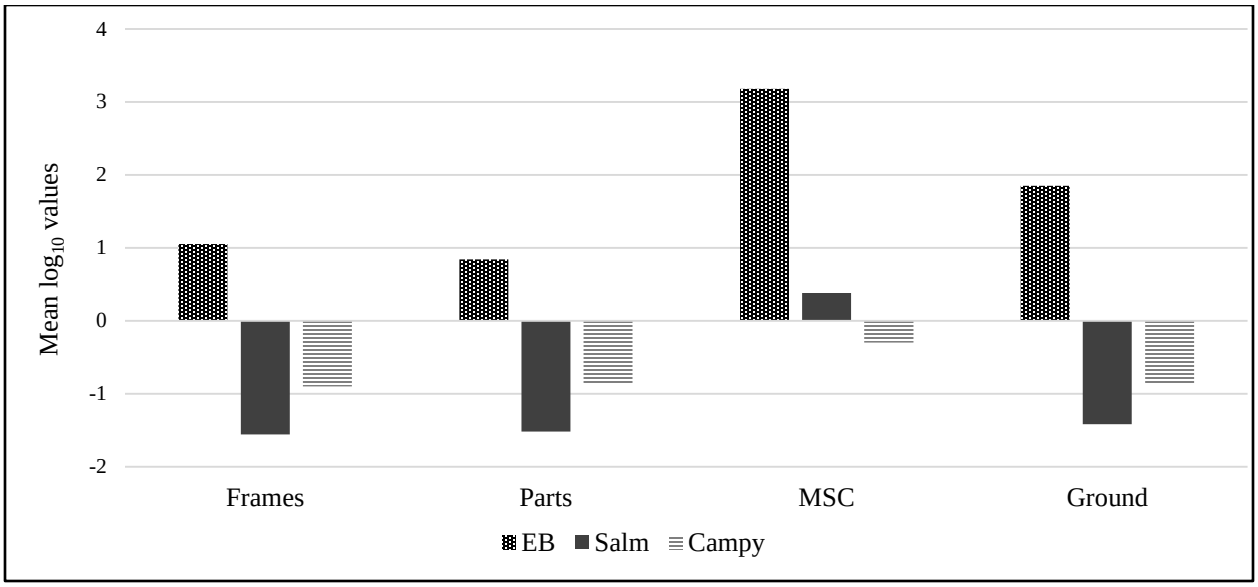
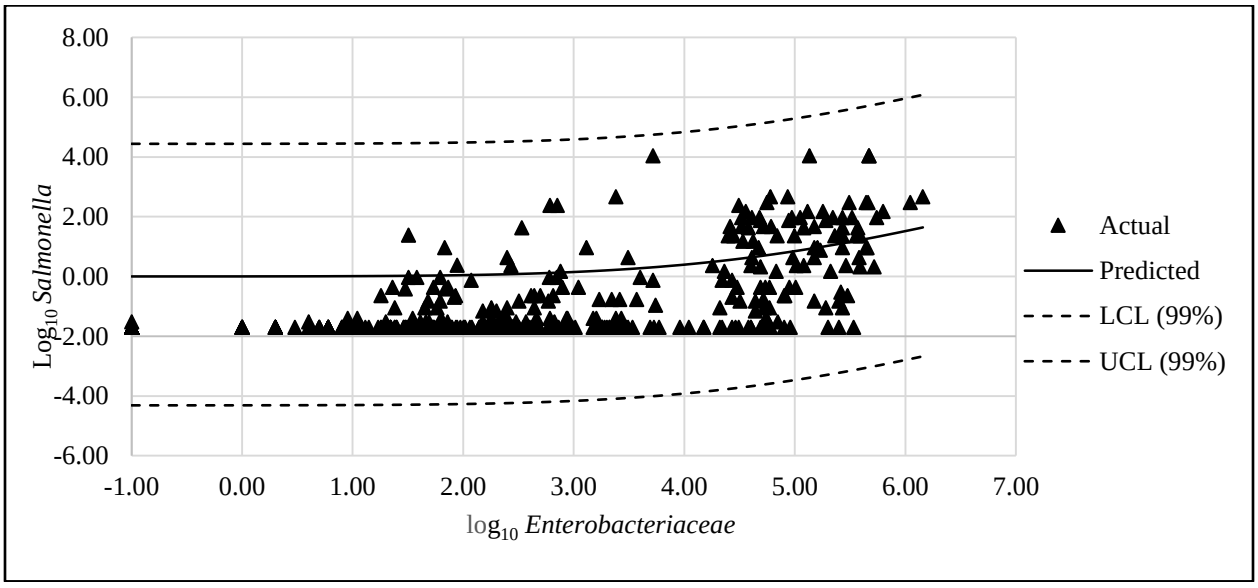
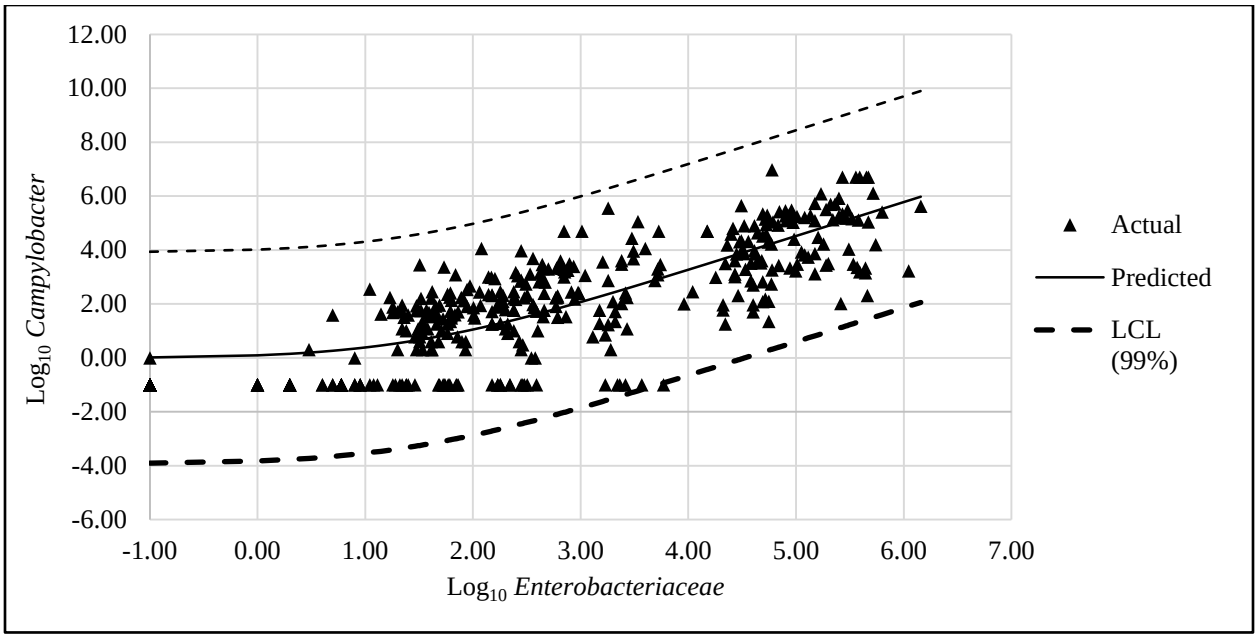


Figure 5.2 Mean $\log_{10}$ values by pathogen type and part type for *Enterobacteriaceae*, *Salmonella* spp., and *Campylobacter* spp. after first processing.



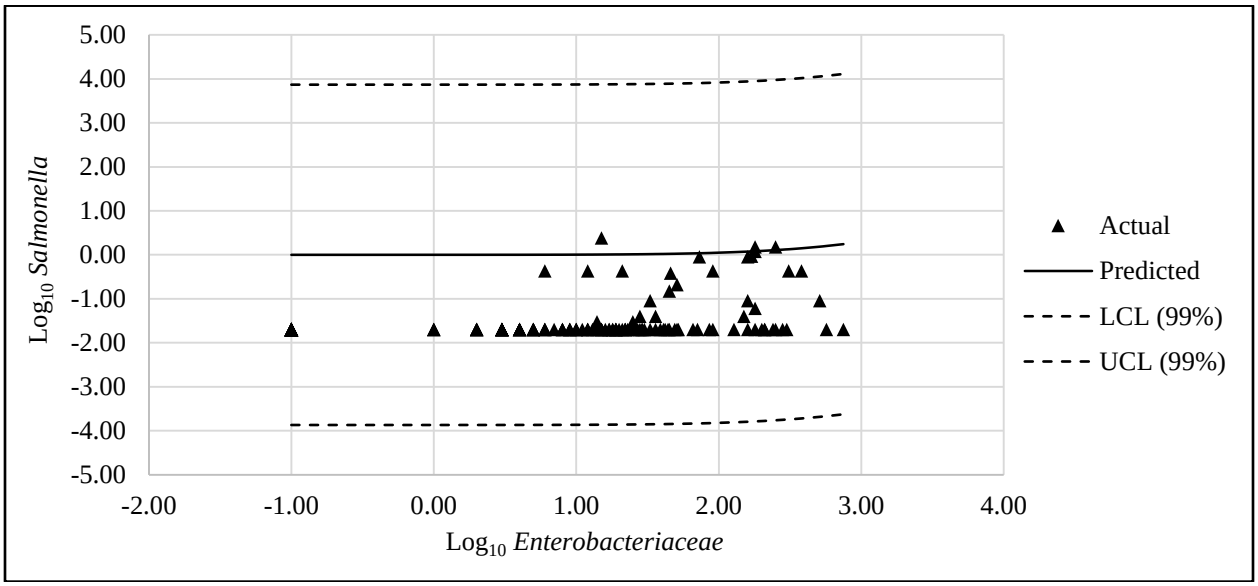
Y'= 0.99x -4.65 (x=*Enterobacteriaceae* count). AIC = 910.02.

Figure 5.3 Mean $\log_{10}$ *Enterobacteriaceae***Salmonella* spp. counts for WBCR's at first processing. Includes actual and predicted counts with 99% confidence intervals.



Y' = 1.26_x – 1.77 (x= *Enterobacteriaceae* count). AIC = 1272.47.

Figure 5.4 Mean $\log_{10}$ *Enterobacteriaceae*Campylobacter* spp. counts for WBCRs at first processing with a Tobit regression. Includes actual and predicted counts with 99% confidence intervals.



Y'=1.42_x -5.03 (x = *Enterobacteriaceae*). AIC = 159.12.

Figure 5.5 Mean $\log_{10}$ *Enterobacteriaceae*Salmonella* spp. counts for parts with a Tobit regression. Includes actual and predicted counts with 99% confidence intervals.

Tobit models (Tobin, 1958) may be used to fit models when there are either left or right censored values that have been arbitrarily assigned due to lack of knowledge about the actual value. As such, the predicted values were based on both the probability of the occurrence of a positive and the predicted value of that positive rather than the predicted value of censored observations. The Tobit (or, censored regression) model is described by the following formula:

$$Y_i = 0 \text{ if } Y_i' \leq 0 \quad (5.1)$$
$$Y_i = Y_i' \text{ if } Y_i' > 0 \quad (5.2)$$

if, Y is the pathogen value, Y' (in this case) is the estimated pathogen value assuming a normal distribution. The predicted values depend upon whether the sample value is above or below the level of censoring and parameters for the distribution above the censored value are determined with a maximum likelihood estimator (MLE) methodology.The relationship between *Enterobacteriaceae* and *Salmonella* spp. for first processing WBCR samples (Figure 5.3) was statistically significant, indicating that variation that occurred in the *Salmonella* spp. counts could be explained by a change in *Enterobacteriaceae* with an Akaike Information Criterion (AIC) measure of fit of 910.02. (The AIC is a relative measure of model quality, with the smaller the score the better the fit.) The relationship between *Campylobacter* spp. and *Enterobactericeae* was also significant (Figure 5.4) for both slope and intercept (AIC = 1272.47). The starting point for the two pathogens was significantly different from the *Enterobacteriaceae* samples, with the *Enterobacteriaceae* samples always exhibiting higher counts.

There was a significant relationship between *Enterobacteriaceae* and *Salmonella* spp. for the parts product (Figure 5.5), with an AIC fit statistic of 159.12. These results, although statistically significant, are most likely the result of almost all 0 counts for both *Enterobacteriaceae* and *Salmonella* spp. on the parts product. Although the Tobit model created

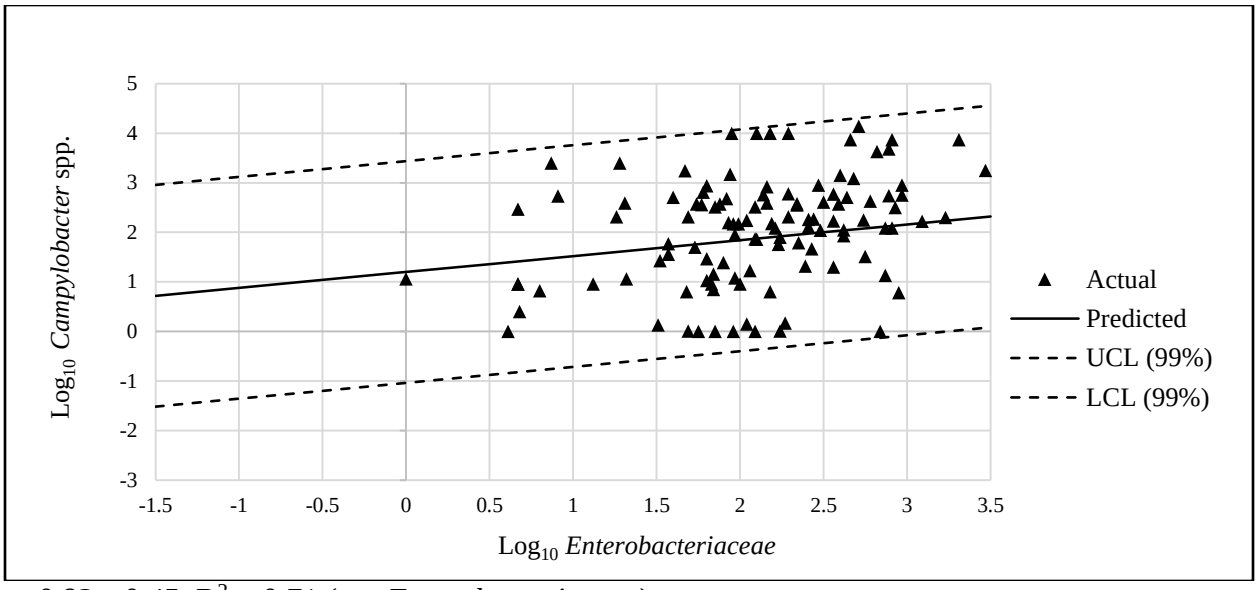
a latent variable for these many zero counts, they nevertheless resulted in very low counts, with little variation in the model.

Table 5.2 Regression analyses of relationship between *Enterobacteriaceae* reduction and pathogen reduction by type: *Salmonella* spp. or *Campylobacter* spp.

Product	*Enterobacteriaceae** *Salmonella* spp. reduction Pr > (t) intercept/model	*Enterobacteriaceae** *Campylobacter* spp. reduction Pr > (t) intercept/model
First Processing WBCR's	0.0003*/0.0961	<0.0001*/<0.0001*

Comparison of reductions through the process from one location to the next in first processing resulted in a significant relationship between *Enterobacteriaceae* and *Campylobacter* spp., with a corresponding significant difference in intercepts (Figure 5.6). An analyses could not be completed for post-debone products due to low sample size. These results differ from prior analyses, where a weak, but significant relationship was found between *Enterobacteriaceae* and *Salmonella* spp. WBCR reductions at first processing (Anonymous, 2015).

Comparison of reductions through the process from one location to the next in first processing was combined due to the similarity in sampling type (WBCR), the increase in sample size, and the applicability of the model to future reduction investigations. Reductions were determined by taking the individual values at one location from the mean of the individual values (n=5) from the preceding location. This methodology, although not without its own inaccuracies, allowed for the investigation to follow the reductions through a specific flock.



$y=0.92_x$ -0.45, $R^2 = 0.71$ (x = *Enterobacteriaceae*).

Figure 5.6 Mean log_{10} *Enterobacteriaceae*Campylobacter* spp. reduction counts for WBCR's at first processing. Includes actual and predicted counts with 99% confidence intervals.

Results exhibited a significant relationship between *Enterobacteriaceae* and *Campylobacter* spp., with a corresponding significant difference in intercepts (Figure 5.6). An analyses could not be completed for post-debone products due to low sample size. These results differ from prior analyses, where a weak, but significant relationship was found between *Enterobacteriaceae* and *Salmonella* spp. WBCR reductions at first processing (Anonymous, 2015).

Analysis of variance results exhibited the highest mean log_{10} counts were at the pre-scald location with counts going down significantly from this location to post-debone products for *Campylobacter* spp. Conversely, for *Enterobacteriaceae* the mean log_{10} count for MSC was significantly lower than that of the pre-scald carcass rinses, but was higher than that of the other products. For *Salmonella* spp., the mean log_{10} count for MSC was not significantly different

from the pre-scald mean $\log_{10}$ count and was significantly higher from all the other product types.

5.5 Conclusion

Employing indicator organisms at food production facilities is often considered a reasonable method of following the behavior of pathogens without the risk of inoculation or the cost of difficult recovery procedures. In this study, product was sampled at multiple poultry processing facilities over a period of 7 months (to capture a representative cross-section of seasonality). These samples were collected at multiple locations in first processing and for several product types at or after debone, with counts being recovered for *Enterobacteriaceae* and *Campylobacter* spp. for each sample collected, with *Salmonella* spp. presence/absence and count (if positive) being recovered from the same sample.

An analysis of variance showed that for *Enterobacteriaceae,* the prescald WBCR had a significantly higher mean $\log_{10}$ cfu/ml than the other parts/locations, with MSC having the second highest mean count, higher than product from locations earlier in the process. A similar pattern was seen for *Salmonella* spp., for which pre-scald and MSC were significantly higher than all other products sampled, but not different from one another. Conversely, *Campylobacter* spp. mean $\log_{10}$ counts decreased successively throughout the process.

Tobit regression analyses were performed on $\log_{10}$ transformed counts to determine if a significant relationship between *Enterobacteriaceae* and either *Campylobacter* spp. or *Salmonella* spp. existed. First processing WBCR samples, taken at the prescald, postpick, prechill, and postchill locations were grouped as the rinse and recovery methodology was the same for these trials. Results revealed a significant linear relationship between $\log_{10}$ transformed

Enterobacteriaceae and *Salmonella* spp. cfu/ml counts and $\log_{10}$ transformed *Enterobacteriaceae* and *Campylobacter* spp. cfu/ml counts. For post-debone samples there was a significant relationship between *Enterobacteriaceae* and *Salmonella* spp. for parts product.

Previous studies exhibited a relationship between *Enterobacteriaceae* and *Salmonella* spp. reduction (from a previous location) in first processing (Anonymous, 2015). Results from this comparison did not show statistically significant results. Further investigation of this association would be a beneficial part of explaining the behavior of *Salmonella* spp. in first processing, specifically after application of interventions.

Results of this analysis should be considered a valid indication that a relationship between *Enterobacteriaceae* and *Campylobacter* spp. needs further exploration. The relative ease of *Enterobacteriaceae* recovery in comparison to that of *Salmonella* spp. enumeration, along with the higher counts of the non-pathogenic organism make it an excellent indicator if the reliability of the relationship can be solidified.

It is suggested that tandem research be completed in a laboratory setting, where *Salmonella* spp. and *Campylobacter* spp. can be inoculated in higher counts than are found in the processing environment. Results from this research were doubtless influenced by the high frequency of 0 results as the low counts found during processing make it difficult to determine if an increase or decrease in one microbe results in a similar change in another, or, if a reduction has transpired through the process.

The recovery process is open to counting error, and, the MPN enumerative method is known to be imprecise. The use of buffered peptone water has been recently brought into question and the use of neutralized buffered peptone water has been suggested to counteract

residual kill in product rinses. In this process, product was allowed to drip for approximately 1 minute, but the efficacy of such a method is still being investigated.

Limitations from the small size of each sampling event (day) by location (n=5) may limit the impact of the strength of the relationship between *Enterobacteriaceae* as an indicator organism for *Salmonella* spp. and *Campylobacter* spp., but the pattern remains and suggests further study. Although the sample sizes were small, the overall impact of randomizing both facility and season worked to seam together a representative sample of the microbial population in a poultry processing environment.

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Chapter 6 Conclusion and Recommendations for Future Research

Poultry processing has been incorrectly impugned in both *Salmonella* spp. and *Campylobacter* spp. foodborne illnesses. Sundry physical and chemical interventions have been introduced at the processing facilities in an attempt to reduce bacterial load on the incoming product. Despite efforts to lower pathogen counts, and evidence that this effort has been successful, reported illnesses have not decreased. As a result, models of the poultry process, from grow-out to retail are needed to make predictions about the efficacy of choice and concentration of intervention in the process.

Quantitative microbial risk analyses (QMRA's) have been used for several decades to infer behavior of microbes in specific situations. Initial microbial risk models focused on single processes and attempted to determine change in microbe load by time, sometimes with an added element (temperature). As models became more complex, and iterative software was developed, multiple processes could be modeled as separate distributions and these models could be connected in a Markov chain, representing an entire process.

The QMRA models developed in this research focused on processes that resulted in three different product types (parts, ground, and MSC). These models are unique in that the current library of quantitative analyses for poultry processing in the samples were followed through poultry processes, instead of utilizing data from multiple data sources. This mechanistic approach resulted in data that was representative of the actual process, rather than assuming the relationship from one location to the next.

QMRA models were separated by season when analysis of variance test (ANOVA) tests exhibited a difference between groups. Results from the QMRA analyses revealed that *Campylobacter* spp. parts (breast and drum) were similar across pathways, with counts going down through the process to almost all 0 counts at endpoint. Ground product for the combined

winter/summer season revealed *Campylobacter* spp. reductions to postchill, than increases occurring at pre-grind, with another decrease in counts for the ground product. The spring season model exhibited the largest reduction across seasons at prechill and postchill, with an uptick in the pregrind product.

Salmonella spp. parts models show the drum process decreasing in counts throughout the entire process, with the breast product decreasing to a mean of 0 and post-pick and staying at that level for the rest of the process. For ground and MSC product the process counts for *Salmonella* spp. tick upward at the grinder or beehive.

Parts products reduce to a level at endpoint that stay within USDA FSIS pathogen standards. Ground and MSC products for both pathogens exhibit an increase in *Salmonella* once the product is homogenized. Reduction simulations that resulted in acceptable levels of these products required very large decreases at prescald or postpick and it is uncertain whether these reductions can be realized in processing or if they will need to be instigated at grow-out.

An exhaustive catalogue of research has been recorded on interventions to reduce *Campylobacter* and *Salmonella* counts at multiple locations in poultry production. Most of these studies were undertaken in a laboratory (in order for inoculation to occur) or were focused on one location at a facility. In this study, multiple locations were considered along with some environmental factors or interventions in the process.

In order to determine the most useful combination of treatments and/or chemical interventions to provide reductions in poultry processing, several factors used in multiple poultry processes were analyzed against the reductions that occurred at the postpick, prechill, and

postchill locations. A stepwise regression analysis was used to provide the relationship among the two or three variables, depending upon the location being analyzed.

Results revealed that a slower line speed resulted in larger mean $\log_{10}$ cfu/ml reductions of *Salmonella* spp. at postpick (although the magnitude of the difference was small), whereas *Campylobacter* spp. reductions were not significantly influenced by this variable. A steam cabinet resulted in higher *Campylobacter* spp. reductions, whereas *Salmonella* spp. exhibited higher mean $\log_{10}$ cfu/ml reductions when no cabinet was present.

At the pre-chill location, reductions were significantly higher for *Campylobacter* spp. when (Peracetic acid) PAA was used, but higher for *Salmonella* spp. reduction when FreshFX® was applied. Results of stepwise analyses revealed that FreshFX® application resulted in higher reductions when the pH was increased for both *Salmonella* spp. and *Campylobacter* spp. For the PAA intervention at prechill, the $\log_{10}$ *Salmonella* spp. reduction was highest at a low concentration, coupled with higher levels of pH.

Chlorine resulted in the higher reductions at the chiller for both *Salmonella* spp. and *Campylobacter* spp. than did the PAA application. The highest *Campylobacter* spp. reductions occurred when pH was high and concentration was low. For *Salmonella* spp., higher reductions occurred when pH went down. FreshFX® resulted in the highest reductions of *Campylobacter* spp. at the finishing chiller, with this intervention not being significantly different statistically from the reductions after application of Cecure® for *Salmonella* spp. at the same location.

As recovery of pathogens during processing can be difficult and costly, use of indicator organisms is accepted to predict the reduction or increase of counts. This study focused on the questions that arise when very low counts, if any, of pathogens are found in a process. One way

to address low frequency and concentration of pathogens in research is use of an indicator organism.

Product was sampled at multiple poultry production facilities in a 7-month period throughout first processing and post-debone and the relationship between *Enterobacteriaceae* and *Salmonella* spp. and *Campylobacter* spp. was analyzed with a least squared regression. For the first processing whole bird carcass rinse (WBCR) samples there was a statistically significant relationship between *Enterobacteriaceae* and *Salmonella* spp., and an even stronger relationship between *Enterobacteriaceae* and *Campylobacter* spp.

Analysis of the relationship between *Enterobacteriaceae* and *Salmonella* spp. or *Campylobacter* spp. counts in first processing WBCR samples resulted in a significant relationship between the *Campylobacter* spp. and the indicator organism. The relationship between *Enterobacteriaceae* and *Salmonella* spp. reductions in first processing was not significant. It is suggested that future research attempts to tie these pathogens with *Enterobacteriaceae* with laboratory studies, where product can safely be inoculated with the pathogens of interest and starting quantities are known.

Future research would benefit from a combination of studies of the efficacy of intervention type, concentration, and pH, along with QMRA modeling to determine not only what reduction is to be predicted based upon intervention, but where in the process that these reductions should take place. Results from the QMRA in this research suggest that reductions needed to meet USDA guidelines at process endpoint for ground and MSC product are actually higher than may be possible if begun at the door of processing environments. For this reason it is suggested that microbial models add a component from the grow-out facilities that could be added to the existing production models in this study.

Sample size was limited in this study due to the significant cost associated with the sampling and recovery design. Replication of this study, with more samples taken per sampling event and location would be beneficial to further definition of the relationship between the different locations in the process and pathogen load. The end result of these studies suggest that QMRA models, coupled with intervention analyses and the use of indicator organisms may be beneficial for understanding the behavior of *Salmonella* and *Campylobacter* in the poultry production process.