Microbiological Evaluation of Commercially Manufactured Animal Feeds to Determine Presence of *Salmonella*, *Escherichia coli* and *Clostridium perfringens*

by

Luis Rolando Munoz Romero

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
August 8, 2020

Keywords: *Clostridium perfringens*, *Clostridium argentinense*, *Bacillus proteolyticus*
feed sampling, feed contamination

Approved by

Kenneth S. Macklin, Chair, Extension Specialist and Professor, Poultry Science
Ruediger Hauck, Assistant Professor, Pathobiology and Poultry Science
Wilmer Pacheco, Assistant Professor and Extension Specialist, Poultry Science

Abstract

Animal feed can potentially expose animals to pathogens which may lead to infection or colonization of the gastrointestinal tract (GIT). Feeds can become contaminated with bacterial pathogens during harvesting/transportation of the ingredients, processing at the feed mill, transportation to the farm or during storage. Pathogenic bacteria have the potential to colonize the GIT of animals leading to either disease of the animal or contamination of the carcasses during processing. The objectives of these studies were to: (1) establish if commercially manufactured animal feed serves as a source of contamination of *Salmonella*, *E. coli* and *Clostridium perfringens*, and (2) to identify the unknown group of bacteria with *Clostridium* like characteristics found in animal feeds and determine its pathogenicity when used in a necrotic enteritis model. Four commercial feed mills and the research feed mill at Auburn University were sampled. A total of 292 samples (132 of feed ingredients and 160 of mixed feeds) were collected over two sampling periods (Nov-Mar and Apr-Oct) from 5 different locations within each feed mill: ingredient receiving, post mixing, post pelleting (hot sample), post cooling and at loadout. All samples were assayed for *Salmonella*, *E. coli* and *C. perfringens* using selective media. Resulting colony forming unit counts were $\log_{10}$ transformed and then analyzed using ANOVA, if significant ($P<0.05$), means were separated using Tukey HSD. Additionally, four typical colonies for Clostridial spp. were isolated from each sample and cultured onto blood agar plates to determine the ability of the isolates to produce typical hemolysis. During this isolation process, an unknown group of bacteria with similar characteristics to those of the *Clostridium* genus were identified. These bacteria were rod-shaped, gram positive, grew under anaerobic conditions, formed spores, produced lecithinase, and some produced double-zone beta hemolysis on sheep blood agar, but, when tested at a molecular level by PCR, they did not have the alpha-toxin gene characteristic for *Clostridium*

perfringens. Consequently, some of those isolates were selected for 16S rRNA gene sequencing analysis for bacterial identification.

For experiment (1), the data shows that the Clostridial spp. counts were significantly higher on the feed ingredients: peanut meal and corn gluten meal (3.91 $\log_{10}$ and 2.61 $\log_{10}$, respectively), and the *E. coli* counts were significantly higher on the feed ingredients: peanut meal and corn meal (4.15 $\log_{10}$ and 2.85 $\log_{10}$, respectively) when compared to the rest of the feed ingredients sampled ($P<0.05$). When the contamination levels of ingredients between feed mills were compared, the corn meal samples collected from feed mills B, C and E had the highest Clostridial spp. counts from both sampling periods. No statistical difference was observed on the *E. coli* counts for corn meal between feed mills during both sampling periods. For soybean meal, the samples collected between Nov-Mar were higher on the *E. coli* counts on feed mill A (1.00 $\log_{10}$), and for the samples collected between Apr-Oct feed mill C was the most contaminated on both, Clostridial spp. counts and *E. coli* counts (2.48 $\log_{10}$ and 2.18 $\log_{10}$, respectively). For the processed samples, it was determined that feed mills B and E had the highest Clostridial spp. counts during both sampling periods in all the stages of processing, and for the *E. coli* counts, only the post mixing and post pelleting stages showed a significant difference being feed mill C (Nov-Mar) the least contaminated on the post mixing stage (1.65 $\log_{10}$) and feed mill B (Apr-Oct) the most contaminated on the post pelleting stage of processing (1.58 $\log_{10}$). Recontamination with *E. coli* after the pelleting process was observed for feed mills A and B. *Salmonella* contamination was not detected in the feed ingredients or processed feed throughout the study. Finally, from the processed feed *C. perfringens* was isolated and confirmed through PCR from two different samples obtained from feed mills A and D. For experiment (2), the sequencing results identified the different isolates as *Clostridium argentinense*—which is a bacterium ubiquitous to soil that can produce a

neuroparalytic toxin— or *Bacillus proteolyticus*—which is a bacterium associated to marine environments including fish processing wastes. The necrotic enteritis model trial showed that strain of *B. proteolyticus* evaluated was not able to induce disease and that the *C. perfringens* isolate recovered from feed mill D was able to induce slight necrotic enteritis lesions (1 score). For future research, greater attention should be placed on *E. coli* and Clostridial spp. contaminated animal feed as a possible source of disease transmission to the animals and the pathogenicity of *Clostridium argentinense* should be evaluated on a live animal trial.

Acknowledgments

First and foremost, I would like to thank God for giving me the strength, knowledge, ability and opportunity to pursue this graduate degree. Without his blessings, this achievement would not have been possible.

I would like to share a special thank you to my parents, Luis Munoz-Paz and Cristina Romero-Aguiluz, for your words of encouragement and wisdom throughout all my life. To my sisters, Jenn and Joho, thank you for all the love and support you have given me over the years.

I would like to share a special thank you to my beloved fiancé, Jesse Evatt, for being my best friend and support throughout this journey of graduate school. Thanks for understanding me and being by my side during those stressful moments.

Finally, I would like to thank everyone who has helped me during these projects. Dr. Ken Macklin, thank you for trusting in me as a graduate student and providing me the opportunity to be part of your lab, I will always be grateful for that. Thank you for your mentorship, support and advice. Dr. Wilmer Pacheco, thanks for being the contact for me to become a graduate student in Dr. Macklin's lab, you are a true Zamorano ambassador. Thanks for all the help provided to make the contacts that allowed us to collect samples in the feed mills and thanks for all the help during the sample collection process. James Krehling, thanks for your friendship, all the technical advice related to the sample processing and help during these projects. Finally, to my lab mates Kaicie, Lasheda, Aiden and all the undergraduate students that work and have worked in lab during my stay, thanks for all of your help making media, preparing samples and spread plating them; without you guys this would have not been possible. I wish you the best in your academic and professional carriers.

Table of Contents

Abstract....2

Acknowledgments....5

List of Tables....8

List of Figures....9

List of Abbreviations....10

Chapter 1.0 Introduction....13

Chapter 2.0 Literature Review....15

2.1 General *Clostridium* Characteristics....15

2.1.1 Structure, Natural Habitat, History and Etiology....15

2.1.2 Categorization of Clostridial Diseases....16

2.1.3 *Clostridium perfringens* (CP)....17

2.1.4 *Clostridium perfringens* Economic Impact....20

2.1.5 *Clostridium argentinense*....21

2.2 *Salmonella* and *E. coli* General Characteristics....22

2.2.1 *Salmonella*....22

2.2.2 *Escherichia coli*....23

2.3 Animal Feed Production and Contamination....23

2.3.1 Grain receiving and Storage....25

2.3.2 Grinding....26

2.3.3 Mixing....26

2.3.4 Pelleting....27

2.3.5 Contamination in Animal Feeds....27

2.4 *Clostridium perfringens* Methods of Control 30

Chapter 3.0 Evaluation of Commercially Manufactured Animal Feeds to Determine Presence of *Salmonella*, *E. coli* and *Clostridium perfringens* 43

Introduction 43

Materials and Methods 45

Results 50

Discussion 56

Conclusion 62

References 63

Chapter 4.0 Isolation and Identification of an Unknown *Bacillus* spp. from Animal Feeds and a Preliminary Study of its Effect When Used in a Necrotic Enteritis Model 75

Introduction 75

Materials and Methods 77

Results 82

Discussion 84

Conclusion 90

References 90

Chapter 5.0 Summary and Conclusion 99

List of Tables

Table 2.1 41

Table 2.2 42

Table 3.1 66

Table 3.2 67

Table 3.3 68

Table 3.4 69

Table 4.1 93

Table 4.2 94

Table 4.3 96

Table 4.4 97

Table 4.5 98

List of Figures

Figure 3.1 70

Figure 3.2 71

Figure 3.3 72

Figure 3.4 73

Figure 3.5 74

List of Abbreviations

°C	degrees Celcius
µL	microliter
µM	micromolar
AFIA	American Feed Industry Association
AFO	animal feeding operation
AGPs	antibiotic growth promoters
APEC	avian pathogenic *E. coli*
BHIB	brain heart infusion broth
BW	body weight
CDC	Centers for Disease Control and Prevention
cm	centimeters
CA	*Clostridium argentinense*
CE	competitive exclusion
CP	*Clostridium perfringens*
CPA	*Clostridium perfringens* alpha-toxin
CPE	*Clostridium perfringens* enterotoxin
CSC	*Clostridium* species counts
CFU	colony-forming unit
CE	competitive exclusion
CO_2	carbon dioxide
DDGS	distiller's dried grains with solubles
DNA	deoxyribonucleic acid

ECC	*E. coli* counts
ERS	Economic Research Service
et al.	et alia (and others)
FAO	Food and Agriculture Organization of the United Nations
FC	feed consumption
FCR	feed conversion ratio
GAO	General Accounting Office
GIT	gastrointestinal tract
GLM	general linear model
HSD	honest significant difference
IFIF	International Feed Industry Federation
mL	milliliter
NAMI	North American Meat Institute
NE	necrotic enteritis
NetB	necrotic enteritis B-like
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
RB	rabbit blood
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SB	sheep blood
SBM	soybean meal
Spp.	species

SNE	subclinical necrotic enteritis
TAE	tris-acetate-EDTA buffer
TSA	tryptic soy agar
TSC	tryptose sulfite cycloserine agar
TTB	tetrathionate brilliant green broth
US	United States
USDA	United States Department of Agriculture
XLT4	xylose lysine tergitol-4

Chapter 1.0 Introduction

Animal feeds are formulated to provide the nutrients that the animals require for their optimal growth and reproduction. However, besides providing the chemical and nutritional constituents to achieve this growth, animal feeds may serve as a vehicle for the transmission of a wide variety of microorganisms to farm animals (Maciorowski et al., 2007). These microorganisms can be acquired from multiple environmental sources during harvest (including dust, soil, water and insects), processing at the feed mill, transportation to or during their storage at the farm (Maciorowski et al., 2006). Some of these microorganisms—i.e. bacterial pathogens—may be potentially harmful and they can produce disease to farm animals in a clinical or subclinical way at the intestinal level (Crump et al., 2002; Tessari et al., 2014). The most common bacterial pathogens found in animal feeds are *Salmonella* (Walker, 1959; Hacking et al., 1978; Kidd et al., 2002; Maciorowski et al., 2006), *Escherichia coli* (Davis et al., 2003; Dargatz et al., 2005) and *Clostridium perfringens* (Wojdat et al., 2006; Tessari et al., 2014; Udhayavel et al., 2017). Each of these bacterial pathogens has the potential to colonize the animal's gastrointestinal tract leading to either disease or contamination of the carcasses during processing at the abattoir. The potential contamination of the carcasses represents a risk for human health because foodborne pathogens can potentially be transmitted through the food chain by poultry products. Therefore, the microbiological evaluation of the feed ingredients and finished feeds becomes a key element to ensure that the feeds are not a source of contamination (Wojdat et al., 2005). Further investigation is warranted to determine the role of animal feeds in the transmission of foodborne pathogens to farm animals. Thus, the objectives of these studies were to establish if manufactured animal feed serves as a source of contamination of *Salmonella*, *Escherichia coli* and *Clostridium perfringens* (Exp. 1) and to identify the unknown groups of bacteria with *Clostridium* like characteristics found

in animal feeds and determine their pathogenicity when used in a necrotic enteritis model (Exp. 2).

References:

Crump, J. A., Griffin, P. M., and Angulo, F. J. 2002. Bacterial contamination of animal feed and its relationship to human foodborne illness. Clinical Infectious Diseases, 35(7), 859-865.

Dargatz, D. A., Strohmeyer, R. A., Morley, P. S., Hyatt, D. R., and Salman, M. D. 2005. Characterization of *Escherichia coli* and *Salmonella enterica* from cattle feed ingredients. Foodbourne Pathogens & Disease, 2(4), 341-347.

Davis, M. A., Hancock, D. D., Rice, D. H., Call, D. R., DiGiacomo, R., Samadpour, M., and Besser, T. E. 2003. Feedstuffs as a vehicle of cattle exposure to *Escherichia coli* O157: H7 and Salmonella enterica. Veterinary Microbiology, 95(3), 199-210.

Hacking, W. C., Mitchell, W. R., and Carlson, H. C. 1978. *Salmonella* investigation in an Ontario feed mill. Canadian Journal of Comparative Medicine, 42(4), 400.

Kidd, R. S., Rossignol, A. M., and Gamroth, M. J. 2002. *Salmonella* and other Enterobacteriaceae in dairy-cow feed ingredients: antimicrobial resistance in western Oregon. Journal of Environmental Health, 64(9), pp. 9.

Maciorowski, K. G., Herrera, P., Jones, F. T., Pillai, S. D., and Ricke, S. C. 2007. Effects on poultry and livestock of feed contamination with bacteria and fungi. Animal Feed Science and Technology, 133(1-2), 109-136.

Maciorowski, K. G., Herrera, P., Kundinger, M. M., and Ricke, S. C. 2006. Animal feed production and contamination by foodborne *Salmonella*. Journal für Verbraucherschutz und Lebensmittelsicherheit, 1(3), 197-209.

Tessari, E. N. C., Cardoso, A. L. S. P., Kanashiro, A. M. I., Stoppa, G. F. Z., Luciano, R. L., and de Castro, A. G. M. 2014. Analysis of the presence of *Clostridium perfringens* in feed and raw material used in poultry production. Food and Nutrition Sciences, 2014.

Udhayavel, S., Ramasamy, G. T., Gowthaman, V., Malmarugan, S., and Senthilvel, K. 2017. Occurrence of *Clostridium perfringens* contamination in poultry feed ingredients: Isolation, identification and its antibiotic sensitivity pattern. Animal Nutrition, 3(3), 309-312.

Wojdat, E., Kwiatek, K., and Kozak, M. 2005. Microbiological quality of animal feedingstuffs in Poland. Bulletin-Veterinary Institute in Pulawy, 49(3), 315.

Wojdat, E., Kwiatek, K., and Kozak, M. 2006. Occurrence and characterization of some *Clostridium* species isolated from animal feedingstuffs. Bulletin-Veterinary Institute in Pulawy, 50(1), 63.

Chapter 2.0 Literature Review

2.1 General *Clostridium* Characteristics

2.1.1 Structure, Natural Habitat, History and Etiology

Clostridium is a genus of the order clostridia; it contains close to 200 species, with only a few species that can be pathogenic to animals or humans (Public Health Agency of Canada, 2011). To understand the impact of Clostridial species in the poultry industry is necessary to understand its general characteristics. The genus *Clostridium* is composed of large, rod-shaped, Gram-positive bacteria, from which most of them produce spores and are resistant to high temperatures and many disinfectants (Jordan, 2001). The majority of the species are anaerobic; however, some species can grow under aerobic conditions or are aerotolerant (Public Health Agency of Canada, 2011). They are ubiquitous worldwide, being found in soil, wetlands, dust, animals, marine and freshwater sediments, and frequently, they are found in low numbers in the intestinal tract of healthy birds and humans (Boulianne et al., 2020). Some of these species are used as probiotics in humans, for example *Clostridium butyricum* (Boulianne et al., 2020). *C. butyricum* benefits extend beyond humans to include broiler chickens, as it has shown potential to be an alternative to antibiotics use to prevent infections with *Salmonella enteriditis* and *Escherichia coli* (Zhang et al., 2016; Zhao et al., 2017). However, some of the Clostridial species are considered to be opportunistic pathogens that causes food spoilage and diseases to both, humans and animals (Jordan, 2001; Boulianne et al., 2020). In the poultry industry, they are referred to as opportunistic pathogens because there are factors that compromise the immunity/health of the flocks and allows these pathogens to induce disease. Typically, these predisposing factors are associated with an inappropriate biosecurity, inappropriate management of the feed and bird density in the houses (Jordan, 2001).

2.1.2 Categorization of Clostridial Diseases

Clostridium species are known for the numerous amounts of toxins they produce, around 18% of all known bacterial toxins are produced by these bacteria (Bailey et al., 2013). Of the 150+ species characterized in the genus, only 35 species are considered pathogenic, and from those 35, only 15 produce potent protein toxins (Popoff and Stiles, 2005). Based on the toxin activity, they can be categorized in three major groups (Boulianne et al., 2020). The first group are "those interfering with the neurotransmitters", like *C. botulinum*, which causes limberneck and it can affect turkeys, chickens, ducks, game birds and mammalian species (Jordan, 2001; Boulianne et al., 2020). This group also includes *C. tetani* which is the causal organism of tetanus and causes prolonged spastic paralysis in humans and the rest of the host that it infects (Coia and Cubie, 1995). The second group are "the clostridial strains proliferating in the intestines" (Boulianne et al., 2020). The species within this group are among the most important agents of enteric diseases in the poultry industry (Cooper et al., 2013). The most common clostridial enteric disease in poultry is necrotic enteritis, which is caused by *Clostridium perfringens* and it can affect multiple avian species. *C. perfringens* in poultry is considered an emerging threat for animal and public health. In general terms, damage to the intestinal tract is caused when large numbers of *C. perfringens* colonize the surface of the intestine and produce high amounts of toxins that result in the degradation of the tips of the micro villi leading to a necrotic process (Al-Sheikhly and Truscott, 1977). However, the specific mechanisms used by *Clostridium perfringens* to colonize the avian intestinal tract and the factors involved in the toxin production are largely unknown. It is generally accepted that predisposing factors in the intestinal tract favor the colonization by *C. perfringens*; mucosal damage caused by coccidiosis being the best-known predisposing factor (van Immerseel et al., 2004). Another example of a pathogen in the second group is *C. colinum*, which causes

ulcerative enteritis in poultry. Its transmitted to the birds through an oral route, and after being ingested by the bird, the bacterium adheres to the intestinal villi, which results in inflammation and ulcers in the small intestine and proximal colon (Cooper et al., 2013). The third and final group comprises "the clostridia localized in the liver and muscle", like *C. novyi, C. septicum, C. sordelli* and *C. chauvoei* (Boulianne et al., 2020). These species belong to the histotoxic clostridia, they have the ability to produce local or systemic lesions and individually or combined they can produce gas gangrene in mammalian and avian species (Popoff, 2016; Silva et al., 2016).

2.1.3 *Clostridium perfringens* (CP)

The pathogenic importance of CP was discovered in the 19th century when it was determined that the bacterium was a causative agent of gas gangrene in humans (Niilo, 1980). From then on, the bacterium has been studied and related to other lethal diseases in different animal species, including poultry (Keyburn et al., 2008). Necrotic enteritis (NE) is one of the most severe diseases caused by this bacterium and it is actually the most important bacterial disease in terms of economic losses for poultry producers (M'Sadeq et al., 2015). Its damage in poultry operations goes to the extent of increasing mortality rates, decreasing live performance of the flocks (lower weights at the end of the cycle), increasing feed conversion ratio (FCR) and increasing medication costs (Hafez, 2011). However, NE is only one of several enteric diseases that may impact the gastrointestinal tract in a negative way. In general, any disturbance of the digestive system (be this mechanical, chemical or biological) that impairs the conversion of feed to meat is referred as an enteric disease (Hafez, 2011). Enteric diseases can be attributed to different pathogens or non-infectious causes that directly impact the intestinal environment (Hafez, 2011). These causes are listed on table 1.

CP is a gram-positive anaerobe spore forming bacterium that is present in the environment as well as the gastrointestinal tracts of all animal species including chickens (Shimizu et al., 2002; Keyburn et al., 2008). On poultry facilities it can be recovered from on-farm environmental samples like walls, fans and dirt surrounding the house. These contaminated environments facilitate the colonization of birds with the bacterium and it has been proposed that the colonization of CP in broiler's small intestine occurs early in the life (Craven et al., 2001). The ability of the bacterium to adapt to oxidative stresses, may explain why CP is easily found and recovered in a myriad of environments (Jean et al., 2004). It represents a risk for human health because it can potentially be transmitted through the food chain by poultry products, and it is one of the most frequently isolated bacterial pathogens in foodborne diseases outbreaks (Buzby and Roberts, 1997). The relevance of this bacterium for the poultry industry is the severity of the damage that it can cause in a flock by being the main causative agent of necrotic enteritis, and this bacterium has been found in all poultry producing countries (Keyburn et al., 2008). A characteristic that differentiates CP from most other bacteria is its ability to produce endospores when environmental conditions become unfavorable for vegetative growth (Bailey et al., 2013). The formation of these endospores not only allows the bacterium to persist under harsh environmental conditions, but it also contributes to its widespread presence (Titball et al., 1999). Endospores have the ability to become active under certain circumstances, and when they go back to their vegetative state, they start producing toxins that can cause damage leading to severe diseases in humans and animals (Bokori-Brown et al., 2011). CP produces the highest number of toxins from the genus and among all known microorganisms, it has the ability to produce at least 17 toxins (Popoff and Stiles, 2005; Bokori-Brown et al., 2011). The toxin production facilitates the degradation of multiple substrates

on the hosts tissue and they are the ones that feed from the host to provide the essential amino acids that the bacterium can't produce (Bailey et al., 2013; Boulianne et al., 2020).

2.1.3.1 *Clostridium perfringens* Toxin Classification

The latest count of toxins known to be produced by CP is 20, and they are considered to be the major virulence factors of the bacterium (Bailey et al., 2013; Revitt-Mills et al., 2015). The toxin production varies from strain to strain, permitting the classification of CP isolates based on the combination of four types of toxins produced (alpha-, beta-, epsilon-, and iota-toxins) which would classify CP strains into five toxinotypes, from A-E (Niilo, 1980; Bailey et al., 2013; Rood et al., 2018). According to literature, the five different toxinotypes (A-E) are able to produce alpha toxin (CPA) but the only toxinotypes that contribute to diseases in poultry are A and C (Petit et al., 1999; Singh, 2017; Boulianne et al., 2020). The difference between the CPA produced by toxinotype A strains and the rest of them is that the ones produced by toxinotype A strains are encoded in the chromosome and the rest of them are encoded on large plasmids. In addition to producing CPA from plasmids these CP produce another major toxin (Petit et al., 1999; Rood et al., 2018). CPA is the toxin primarily associated with diseases such as NE and gangrenous dermatitis in the poultry industry (Niilo, 1980; Keyburn et al., 2008). The way in which CPA causes damage in the host is by hydrolyzing lecithin present in the lipoproteins of the cell membrane, leading to hemolysis, necrosis or death depending on the number of toxins and accessibility to the tissues (Niilo, 1980; Ispolatovskaya, 2012). The current toxinotyping scheme does not include two types of toxins within the 20 that are now known to be produced by CP, these toxins are enterotoxins (CPE) and Necrotic Enteritis Toxin B-like (NetB) (Keyburn et al., 2008; Brynestad and Granum, 2002; Rood et al., 2018). CPE has been reported to produce food poisoning in humans causing an acute intestinal upset, with the ileum being the region that appears to be

most sensitive to this toxin (McClane, 1996; Brynestad and Granum., 2002). The way on which CPE causes damage is by affecting the tips of the villi of the epithelial cells in the gastrointestinal tract which causes fluid secretion into the lumen leading to diarrhea and electrolyte loss (McClane, 1996; Rood, 1998). Finally, research on NetB implies that this toxin is an important virulence factor for causing necrotic enteritis in chickens (Keyburn et al., 2008). The plausible mechanism proposed on NetB relies on a different CP gastrointestinal population between sick and healthy birds, where the healthy birds express mixed populations of CP with low percentage of NetB clones and the diseased birds express a dominant NetB expressing clone (Keyburn et al., 2010). However, follow up studies have demonstrated that it is possible to induce NE with CP strains that have tested negative for NetB, giving a reasonable doubt about the role of the toxin inducing to NE (Cooper and Songer, 2010). Based on the relatively recent discovery of these last two toxins produced by CP strains (CPE and NetB), an expansion of the CP toxin-based scheme has been proposed and on the review is suggested that the toxinotype F consist of the isolates that produce CPE but not beta- epsilon- or iota- toxins and the toxinotype G consist on those isolates that produce the NetB toxin (Rood et al., 2018).

2.1.4 *Clostridium perfringens* Economic Impact

The economic cost of CP infections is extremely elevated due to its ability to cause varied infections in humans and in animals (English, 2015). In humans, CP is one of the most common causes of foodborne illness in the United States and it is estimated that it causes nearly 1 million cases of foodborne illness every year (Hoffmann et al., 2015). When causing foodborne disease, it is responsible for about 440 hospitalizations and 25 deaths each year (Hoffmann et al., 2015). According to the United States Department of Agriculture (USDA) the estimated annual cost for human associated infections with CP is $343 million [Economic Research Service (ERS), 2014).

Research has been done to quantify the impact of CP in the animal industry, particularly in the poultry industry. A study showed that subclinical necrotic enteritis (SNE) impaired chickens to reach their average body weight by 12% when compared to healthy birds, additionally the sick birds had an increased FCR by 10.9%; these factors impacted the production efficiency and production costs and the average observed loss was of $0.29/bird on all animals infected with SNE (Skinner et al., 2010). If analyzed in a worldwide scale, in the year 2000, a study showed that the impact of SNE in poultry was around $2 billion, however a reevaluation of this value was done on 2015 and the estimated cost for subclinical necrotic enteritis was within the 5 – 6 billion/year range worldwide (Wade and Keyburn, 2015).

2.1.5 *Clostridium argentinense*

C. argentinense was first isolated by Gimenez and Ciccarelli (1969) from a cornfield in Argentina. Initially, this bacterium was proposed to be a prototype for type G *Clostridium botulinum* due to its ability to produce a botulinal-like toxin (Gimenez and Ciccarelli, 1970). However, it was distinguished from *C. botulinum* due to the inability of this organism to metabolize carbohydrates (specifically glucose), the negative lipase reaction and the volatile fatty acids by-products produced in peptone-yeast extract-glucose broth cultures (Ciccarelli et al., 1977). *C. argentinense* is a motile, peritrichous, anaerobic, gram positive rod that may or may not produce spores (Gimenez and Ciccarelli, 1970). The organism has the ability to produce beta-hemolytic colonies on rabbit blood agar, and it has weak or absent hemolysis on sheep blood agar (Suen et al., 1988).

According to Suen et al. (1988), not all strains of *C. argentinense* have the ability to produce a neuroparalytic toxin, making its pathogenicity in laboratory animals to be variable. However, those strains of *C. argentinense* that produce neurotoxins, display similar clinical signs

and symptoms to those caused by botulinal toxins (types A-F) (Ciccarelli et al., 1977). In chickens, after oral and subcutaneous administration, the pathogenicity of the botulinal-like toxin produced by *C. argentinense* were muscular weakness in the legs and neck, paralysis (as the illness progressed), limberneck and death (Ciccarelli et al., 1977). In humans there are no reports of illness associated to *C. argentinense*, however, it has been isolated at necropsy from four adults and an 18-week-old infant that died suddenly without any pathological evidence to account as the cause of death (Sonnabend et al., 1981).

2.2 *Salmonella* and *E. coli* General Characteristics

2.2.1 *Salmonella*

Salmonella is a group of bacteria known to cause disease in humans and animals, they belong to the family Enterobacteriaceae. In the United States alone, it is estimated that *Salmonella* serovars cause more than one million salmonellosis cases every year with more than 19,000 hospitalizations and 378 deaths (ERS, 2014). Additionally, infections with these organisms have an estimated financial cost of $3.6 billions per year due to medical expenses, loss of productivity and premature death (ERS, 2014).

Salmonella are gram negative, rod-shaped, facultative anaerobic bacteria with a peritrichous flagella that facilitates the movement of the bacteria throughout the digestive tract of the animals (D'Aoust et al., 2007; Chadwick, 2017). *Salmonella* serovars are widely distributed throughout the environment and they are capable of producing a spectrum of diseases in humans (gastroenteritis, enteric fever, septicemia) and animals (enteritis, septicemia, abortion and asymptomatic carriage) (Agbaje et al., 2011). The genus is classified in two major species: *S. bongori* and *S. enterica*, from which the last is the largest of the two species—containing 2,500+ serovars defined on the basis of the serologic identification of somatic (O) and flagellar (H)

antigens—and it can be further divided into six subspecies: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV) and *indica* (VI) (Grimont and Weill, 2007). From the 2,500+ serovars, only a limited number are capable to induce to infection in humans and animals, being *S.* Typhimurium and *S.* Enteriditis the most common causes of human salmonellosis due to their ability to invade different hosts without causing illness (Herikstad et al., 2002; Singh, 2013).

2.2.2 *Escherichia coli*

E. coli is gram negative, facultative anaerobe, rod-shaped bacterium from the Enterobacteriaceae family (Bennett et al., 2014). It is the most common facultative anaerobe species found in the gastrointestinal tract of humans and animals (Bennett et al., 2014). It lives as a commensal organism that coexists with its host (Kaper et al., 2004). Within the gastrointestinal tract, *E. coli* resides in the lumen of the colon and its excreted into the environment in fecal matter. Even though that most strains of *E. coli* are non-pathogenic and resides harmlessly within the gastrointestinal tract, some strains have the potential to cause disease producing diarrhea, urinary tract infections and sepsis/meningitis (Kaper et al., 2004). In poultry, only certain strains are more capable to cause disease, and those strains are referred to as avian pathogenic *E. coli* (APEC). These APEC are mostly associated with extra-intestinal infections causing damage in the respiratory tract or systemic infections (Dho-Moulin and Fairbrother, 1999).

2.3 Animal Feed Production and Contamination

The intensification of agriculture over the last several decades has created thousands of Animal Feeding Operations (AFO) across the US. The AFO are agricultural enterprises where animals are kept and raised in confined situations. Confinement has enabled more economical production by increasing efficiencies, minimizing waste and reducing costs for the animal protein producers; however, it has also amplified the level of exposure of the animals to pathogens due to

the proximity of higher numbers of animals (Maciorowski et al., 2006). These higher animal numbers also create a concern over the animal's feed since more animals can become exposed if any type of contamination occurs in the feed.

Annually, over 1 billion metric tons of feed are estimated to be produced worldwide to supply feed for livestock (bovine and ovine), poultry and swine species [International Feed Industry Federation (IFIF), 2020]. In the United States, other than the feed mills, the industries that play important role in the expansion of feeds are rendering plants and protein blenders [General Accounting Office (GAO), 2000]. Feed mills combine animal and plant-based ingredients to produce a diet specific to the species of animal of interest (GAO, 2000). Rendering plants transform meat scraps, slaughter by-products and animals that are not suitable for human consumption into animal feed ingredients and protein blenders combine vegetable and animal-based protein ingredients into animal feeds (GAO, 2000). In the United States, a total of 5,715 animal feed mills produce over 236 million tons of finished feed per year [American Feed Industry Association (AFIA), 2020]. At the time of this writing, Alabama was No. 2 state in the US broiler production, processing approximately 21 million birds per week, which consume annually around 26.1 million tons of corn and 13.6 million tons of soybean meal (SBM) for poultry feed [Alabama Farmers Federation (Alfafarmers) 2019].

When compared to other animal protein industries, the poultry industry is the major consumer of grain-based complete diets; therefore, the production of this feed is a crucial point to ensure that no bacterial pathogens are being transmitted through the feed. Feed by itself, is the most important input of the poultry industry that can expose the birds to a wide variety of factors through the gastrointestinal tract (Yegani and Korver, 2008). Typical diets for avian species are produced using corn as energy source, SBM as a protein source and premixes to supplement

vitamins and minerals on the diet. Recently, the inclusion of wheat and barley has been evaluated to substitute corn in poultry diets, but no satisfying results have been observed due to the presence of non-starch polysaccharides in these cereals that are not well digested by chickens (Lesson et al., 2000). Additionally, other studies have been performed to compare the bacterial proliferation between poultry diets developed with corn, wheat or barley as energy sources and the findings showed that the diets developed with wheat and barley had a significantly higher ($P<0.05$) CP proliferation when compared to corn-based diets (Annett et al., 2002). At the feed mill, the ingredients are exposed to different processes such as grain receiving, grinding, mixing and pelleting and there are many opportunities for pathogens to contaminate feed ingredients or re-contaminate finished feeds during the processing (Maciorowski et al., 2006).

2.3.1 Grain receiving and Storage

Corn is typically the energy source used for poultry diets around the world. Commercial animal feed mills rely on grain reception policies to ensure the quality before unloading it into the bins; usually grains are tested for moisture, mycotoxins, foreign material and mold damage [Food and Agriculture Organization of the United Nations (FAO), 1994]. Ingredient testing ensures the quality of cereal grains and other feed ingredients prior to animal feed production. During storage, temperature, moisture, carbon dioxide, oxygen, microorganisms, insects, rodents, birds, geographical location can influence the quality of stored grains (Jayas and White, 2003). Other studies have suggested that deterioration in grains due to microbial activities are related with the level of moisture and temperature at which the grains are stored (White, 1995). The preservation of the nutritional content and the prevention of growth of pathogens are indispensable to obtain a quality feed for the food animal industries.

During the storage, grains may be exposed to damage and deterioration due to cracks, broken seed coat, presence of foreign material, insect infestation and proliferation of microbial contaminations (Tuite and Foster, 1979). Safe storage guidelines can be found on the internet to understand how to preserve the shelf quality of the stored grains, an example of this guidelines is provided in detail by Jayas et al. (1994). The microbial content and nutritional quality of the feed ingredients prior to receiving and storage can determine feed safety and quality through the rest of the feed manufacturing processes. During storage, the major microbial deterioration on the grains is produced by fungal growth and mold-induced mycotoxins (Tuite and Foster, 1979).

2.3.2 Grinding

Grinding is the mechanical action of reducing the particle size of the grain. Grains are ground prior to mixing with the objective of increasing their surface area to obtain an improved rate of digestion, decrease segregation and mixing problems, and to facilitate further processes during the feed manufacturing such as pelleting or extrusion (Behnke, 1996). Particle size of the feed provided to the avian species plays an important role in the development of the digestive system and the health of the intestinal tract of the birds. Feed, water or any other material that the birds ingest may contain different nutrients and microorganisms that could be beneficial or harmful to the birds. This factor, according to Yegani and Korver (2008), makes the digestive tract of the chicken the major site of potential exposure to pathogens, being *Salmonella*, *E. coli* and CP some of the pathogens that can cause intestinal tract damage that is going to lead to economic losses due to poor feed efficiency, higher mortality and increased medication costs (Skinner et al., 2010).

2.3.3 Mixing

According to Behnke (1996) the mixing process is one of the most critical and essential operation during the feed elaboration process, because the lack of the proper mixing can lead to a

reduced diet uniformity. In a subsequent study, Behnke and Beyer (2004), stated that nutrient uniformity is important to optimize the animal's growth, production and health through the supply of nutrients and feed additives at desired concentrations. The uniformity of the feed becomes more critical at early stages of life of the chickens and chicks because they only consume few grams of feed and it is necessary to provide all the essential nutrients in the adequate amounts to ensure the optimal development of the birds (Ensminger et al., 1990).

2.3.4 Pelleting

The process of pelleting has become an important process to the feed industry due to its multiple benefits on the animal's performance (Behnke, 1996). The integrated poultry and swine companies have recognized the benefits of pelleting feeds and have increased the amount of feed that is being pelleted (Zang et al., 2009). Pelleting per se, is a process of forcing and shaping bulk material through a die in order to improve the physical condition and nutritional quality of the feed (Kokić et al., 2013). The influence of pelleting on the pathogenic bacterial content of the feed has been studied and according to the results of these investigations the number of pathogenic bacteria can be reduced or eliminated depending on the type of the bacteria (Furuta et al., 1980) and pelleting conditions. Enteric bacteria such as *Salmonella* and *E. coli* can be eliminated by pelleting if certain temperatures and retention times during feed conditioning are met (Akaike et al., 1970). For *Clostridium* species the pelleting process has little effect since these species are thermo-resistant and spore forming (Prió et al., 2001).

2.3.5 Contamination in Animal Feeds

Animal feeds are usually formulated to provide the nutrients that the animals require for their optimal development, production and reproduction. The formulation of these feeds relies on a wide range of ingredients including cereal grains, animal by-products, milling by-products,

mineral supplements, vitamin supplements and additives. The ingredients to be used on each feed depend on the animal species that it is intended for. Table 2 provides a list of some ingredients used in the feed industry in the US. Due to the multitude of origins of the feed ingredients, their inclusion into the animal feed can result in the presence of a range of biological, chemical, and other etiological agents that may affect the quality and safety of protein foods from animal origin —i.e. meat and eggs in the poultry industry— and pose potential risks of human foodborne illness transmission (Crump et al., 2002; Sapkota et al., 2007).

Additionally, animal feeds may serve as a carrier of a wide variety of microorganisms, chemical substances or other etiologic agents that represent a risk when the feed is given to the animals due to the potential transmission of disease (Crump et al., 2002; Sapkota et al., 2007). When referring to the contamination of the feed with bacterial pathogens the potential contamination may occur at the harvest/transportation of the feed ingredients, processing at the feed mill or at any point during storage or transportation to the farm (Maciorowski et al., 2006). There is substantial evidence that animal feeds are often contaminated with bacteria such as *Salmonella* (Walker, 1959; Hacking et al., 1978; Kidd et al., 2002; Maciorowski et al., 2006) and *E. coli* (Davis et al., 2003; Dargatz et al., 2005). These studies show that the contamination of the animal feeds is related to the addition of a contaminated ingredient—i.e. bone meal, fish meal, meat meal, feather meal—during mixing process in the feed mill. However, cereal grains also represent a source of potential contamination due to the diverse microbial population of Clostridial spp. (*C. perfringens* and *C. botulinum*), *Listeria monocytogenes*, *E. coli* and *Salmonella* (Maciorowski et al., 2007) that might be present. Contaminated ingredients are not the only source of contamination in feeds, additional contamination can occur if the finished feeds come in contact with any environment that harbors *Salmonella*, *E. coli* or any other bacterial pathogen or if the

finished feed is disturbed by insects, wild birds or animals that harbor these bacteria (Maciorowski et al., 2006; Sapkota et al., 2007).

2.3.5.1 Animal Feed Contamination with Clostridial spp.

As stated previously, the presence of clostridial spp. in animal feed represents a major concern for the animal protein producers (Maciorowski et al., 2007). The *Clostridium* spp. found in the feed that are of major concern are CP and *C. botulinum* due to their potential to cause illness in humans and animals (Maciorowski et al., 2007). From the multiple environments that clostridia can be recovered, soil is thought to be their primary habitat; it's thought that the clostridia in the soil ends in the plants and crops carried through wind, rain or mechanical harvesting of the grains, becoming a potential source of contamination for the live animals, feed ingredients and animal feeds (Wojdat et al., 2006; Tessari et al., 2014). The ingredients in the feed processing plants that have been related to show the highest numbers of CP are those originated from processed animal proteins such as: fish meals, meat meal, feather meal or organ meal (Wojdat et al., 2006; Tessari et al., 2014). This was confirmed in a more recent study (Udhayavel et al., 2017) were the highest levels of contamination of ingredients with CP were found in animal protein sources, the rate of contamination of 55.26%, 44.83% and 42.86% for fish meal, bone meal and meat and bone meal, respectively. At the same time, this study showed that finished layer animal feed had 22.58% of contamination with CP (Udhayavel et al., 2017). Other research shows that CP contamination can occur in finished animal feeds for species other than poultry, indicating levels of contamination with CP of 46.2%, 42.9 and 42.2% for poultry, cattle and swine finished feeds, respectively (Wojdat et al., 2006).

2.4 *Clostridium perfringens* Methods of Control

For decades, antibiotics have been used to treat and prevent bacterial infections in food-producing animals (Schwarz et al., 2001). Besides its therapeutic and metaphylactic benefits, the usage of antibiotics has shown to be of economic advantage for producers because when administered in low doses they help to improve the weight gains and FCR (Izat et al., 1990; Schwarz et al., 2001). Typically, the addition of low doses of antibiotics has taken place at the feed mills, adding them as an additive in the animal feeds (van Immerseel et al., 2009). However, discoveries of antibiotic-resistant bacteria in animals created a global concern not only due to the spread of antibiotic-resistant genes through the animal feed, but due to the wider dissemination of waste materials containing antibiotic residues from animal operations to the environment (van Immerseel et al., 2009). For the specific case of CP, the antibiotic resistance is of special concern, due to the capability of the bacterium to produce the lethal toxins that have been previously described that can affect animals and humans.

As expected, the ban of antibiotics used in feeds as growth promoters has had a negative economic impact due to higher FCR experienced by the food animals, however, some unexpected effects have also been noted within the poultry industry (Grave et al., 2004; Feighner and Dashkevicz, 1987). One of these effects is the inevitable change of the microbiome of the gastrointestinal tract of poultry species that has made the birds more susceptible to diseases such as NE (Knarreborg et al., 2002). Therefore, different approaches other than the use of antibiotics to prevent and control CP infections in poultry have been recommended, and they can be classified as measures to apply to the live birds or measures to reduce their exposure to pathogens (i.e. use of organic acids in feed to reduce pathogen presence). For the measures to be applied in live birds, the first intervention strategy to prevent contamination with CP in the birds would be an effective

prevention of coccidiosis, which is considered a predisposing factor for NE (described earlier). The mechanism by which coccidiosis induces NE is still not fully understood. However, two possible mechanisms have been proposed, the first is that the coccidial infection predisposes the birds to NE and the second relies on the severe damage caused to the intestinal epithelium by coccidia that allows the CP infection to start NE (Williams, 2002). However, the poultry industry can prevent coccidia by using anticoccidial vaccines, which contain live attenuated *Eimeria* oocysts, which provide protection against coccidia infection and indirectly protects the birds from the toxicity of CP (Williams et al., 2003). A second intervention strategy that has been described to reduce the incidence of CP infections is the use of competitive exclusion (CE) products. The concept behind CE is to administer material from the gastrointestinal tract of adult healthy birds into the new-hatched chickens to help the establishment of beneficial intestinal flora (Nurmi and Rantala, 1973). During a study to determine the effect of CE over the incidence of NE it was found that CE treatments not only displayed lower incidence of NE and lower caecal carriage of CP, but the grow-out cycle was achieved with less total mortality and an increased carcass yield (Elwinger et al., 1992).

On the other end, the measures to reduce exposure of the birds to CP include the use of organic acids (propionic acid, formic acid, succinic acid, fumaric acid, malic acid, lactic acid, caprylic acid) that can be added to the poultry feed to ensure that the bacterial exposure is reduced. It is believed that organic acids can be a suitable substitute to growth promoter antibiotics used in feeds, this due to their properties to preserve and protect the feeds from microbial deterioration, improve daily weight gains and FCR in the animals and significantly reduce the presence of pathogens in the intestinal tract (Roth and Kirchgessner, 1998; Skřivanová et al., 2006). The mode of action of the organic acids in the digestive system is not fully understood. However, it is

believed that the benefits perceived come from the ability of the organic acids to reduce the pH of the diet, reducing its buffering capacity and supporting a more efficient breakdown of proteins in the stomach, which translates into a better protein digestion on the intestinal tract (Roth and Kirchgessner, 1998). The antimicrobial effects are attributed to the undissociated form of the organic acids, when they penetrate the bacterial cell membrane they dissociate within the cell creating stress conditions that disrupt their protein synthesis making them unable to replicate, therefore lowering the pathogen presence in the intestinal tract (Roth and Kirchgessner, 1998; Lück and Jager, 2013). CP has been reported to be susceptible to caprylic, capric, lauric, miristic, oleic, linoleic and citric acids at different concentrations (Skřivanová et al., 2006). Other prevention strategies include dietary supplementation with probiotics to reduce the birds' exposure to pathogens. Hofacre et al. (1998) reported a lower incidence of NE when probiotics were fed to new-born chickens, however they didn't create a barrier against the infection.

Proper management techniques, adequate composition of the feed, enhanced biosecurity, clean sources of feed and water are also vital to reduce the exposure of birds to infections associated with CP.

References:

American Feed Industry Association (AFIA), 2020. Feed industry statistics. Accessed February 2020. Available at https://www.afia.org/feedfacts/feed-industry-stats/

Agbaje, M., Begum, R., Oyekunle, M., Ojo, O., and Adenubi, O. 2011. Evolution of *Salmonella* nomenclature: a critical note. Folia Microbiologica, 56(6), 497-503.

Akaike, Y., Nakajima, T., Nakayama, N., Osada, H., and Kashiwazaki, M. 1970. Contamination of aerobic bacteria in materials for feed and commercial feed for swine. SPF Swine, 1, 21-26.

Alabama Farmers Federation (Alfafarmers), 2019. Alabama Poultry facts. Accessed July 2019. Available at http://alfafarmers.org/programs/divisions/commodities/poultry/

Al-Sheikhly, F., and Truscott, R. B. 1977. The interaction of *Clostridium perfringens* and its toxins in the production of necrotic enteritis of chickens. Avian Diseases, 21 (2), 256-263.

Annett, C. B., Viste, J. R., Chirino-Trejo, M., Classen, H. L., Middleton, D. M., and Simko, E. 2002. Necrotic enteritis: effect of barley, wheat and corn diets on proliferation of *Clostridium perfringens* type A. Avian Pathology, 31(6), 598-601.

Bailey, M.A., Macklin, K.S. and Krehling, J.T. 2013. Use of a Multiplex PCR for the Detection of Toxin-Encoding Genes *netB* and *tpeL* in Strains of *Clostridium perfringens*. International Scholarly Research Notices, 2013.

Bennett, J., Dolin, R., and Blaser, M. 2014. Mandell, Douglas, and Bennett's principles and practice of infectious diseases: 2-volume set (Vol. 2). Elsevier Health Sciences.

Behnke, K. C. 1996. Feed manufacturing technology: current issues and challenges. Animal Feed Science and Technology, 62(1), 49-57.

Behnke, K. C., and Beyer, R. S. 2004. Effect of feed processing on broiler performance. Doctoral dissertation, Dissertation, Kansas State University, Manhattan.

Bokori-Brown, M., Savva, C. G., da Costa, S. P. F., Naylor, C. E., Basak, A. K., and Titball, R. W. 2011. Molecular basis of toxicity of *Clostridium perfringens* epsilon toxin. The FEBS Journal, 278(23), 4589-4601.

Boulianne, M., Uzal, F., Opengart, K. 2020. Clostridial diseases. Ch. 22, 966-985 in Diseases of Poultry. Swayne, D.E., 14th Edition. John Wiley & Sons, Inc.

Brynestad, S., and Granum, P. E. 2002. *Clostridium perfringens* and foodborne infections. International Journal of Food Microbiology, 74(3), 195-202.

Buzby, J.C. and Roberts, T., 1997. Economic costs and trade impacts of microbial foodborne illness. World Health Statistics Quarterly 1997; 50 (1/2): 57-66.

Chadwick, E. 2017. Colonization sites of *Salmonella Enteritidis* and *Heidelberg* in broilers when exposed continuously in feed or day of hatch. Electronic Theses and Dissertations. Auburn University.

Ciccarelli, A., Whaley, D., McCroskey, L., Gimenez, D., Dowell, V., and Hatheway, C. 1977. Cultural and physiological characteristics of *Clostridium botulinum* type G and the susceptibility of certain animals to its toxin. Applied and Environmental Microbiology, 34(6), 843-848.

Coia, J., and Cubie, H. 1995. *Clostridium tetani*. In the Immunoassay Kit Directory (pp. 688-690). Springer, Dordrecht.

Cooper, K. K., and Songer, J. G. 2010. Virulence of *Clostridium perfringens* in an experimental model of poultry necrotic enteritis. Veterinary Microbiology, 142(3-4), 323-328.

Cooper, K. K., Songer, J. G., and Uzal, F. A. 2013. Diagnosing clostridial enteric disease in poultry. Journal of Veterinary Diagnostic Investigation, 25(3), 314-327.

Craven, S. E., Stern, N. J., Bailey, J. S., and Cox, N. A. 2001. Incidence of *Clostridium perfringens* in broiler chickens and their environment during production and processing. Avian Diseases, 45(4), 887-896.

Crump, J. A., Griffin, P. M., and Angulo, F. J. 2002. Bacterial contamination of animal feed and its relationship to human foodborne illness. Clinical Infectious Diseases, 35(7), 859-865.

D'Aoust, J. Y., and Maurer, J. 2007. *Salmonella* species. In Food Microbiology: Fundamentals and Frontiers, Third Edition (pp. 187-236). American Society of Microbiology.

Dargatz, D. A., Strohmeyer, R. A., Morley, P. S., Hyatt, D. R., and Salman, M. D. 2005. Characterization of *Escherichia coli* and *Salmonella enterica* from cattle feed ingredients. Foodbourne Pathogens & Disease, 2(4), 341-347.

Davis, M. A., Hancock, D. D., Rice, D. H., Call, D. R., DiGiacomo, R., Samadpour, M., and Besser, T. E. 2003. Feedstuffs as a vehicle of cattle exposure to *Escherichia coli* O157: H7 and Salmonella enterica. Veterinary Microbiology, 95(3), 199-210.

Dho-Moulin, M., and Fairbrother, J. M. 1999. Avian pathogenic *Escherichia coli* (APEC). Veterinary Research, BioMed Central, 30 (2-3), 299-316.

Economic Research Service (ERS), U.S. Department of Agriculture (USDA), 2014. Cost Estimates of Foodborne Illnesses. Accessed January 2020. Available at https://www.ers.usda.gov/data-products/cost-estimates-of-foodborne-illnesses/cost-estimates-of-foodborne-illnesses/#Pathogen

Elwinger, K. A., Schneitz, C., Berndtson, E., Fossum, O., Teglöf, B., and Engstöm, B. 1992. Factors affecting the incidence of necrotic enteritis, caecal carriage of *Clostridium perfringens* and bird performance in broiler chicks. Acta Veterinaria Scandinavica, 33(4), 369-378.

English, K.G. 2015. Effect of biosecurity and management practices on the prevalence of *Salmonella, Campylobacter*, and *Clostridium perfringens* in a poultry production system. Electronic Theses and Dissertations. Auburn University.

Ensminger, M. E., Oldfield, J. E., and Heinemann, W. W. 1990. Feeds and nutrition digest: formerly, Feeds and nutrition--abridged.

Feighner, S.D. and Dashkevicz, M.P., 1987. Subtherapeutic levels of antibiotics in poultry feeds and their effects on weight gain, feed efficiency, and bacterial cholyltaurine hydrolase activity. Applied and Environmental Microbiology, 53(2), 331-336.

Food and Agriculture Organization of the United Nations (FAO) (1994). Grain storage techniques. Accessed June 2019. Available at http://www.fao.org/3/T1838E/T1838E00.htm#Contents

Furuta, K., Morimoto, S., and Sato, S. 1980. Bacterial contamination in feed ingredients, formulated chicken feed and reduction of viable bacteria by pelleting. Laboratory Animals, 14(3), 221-224.

General Accounting Office (GAO), 2000. Food safety: controls can be strengthened to reduce the risk of disease linked to unsafe animal feed. Accessed February 2020. Available at https://books.google.com/books?id=BRw0AAAAIAAJ&printsec=frontcover&source=gbs_ge_summary_r&cad=0#v=onepage&q=rendering&f=false

Gimenez, D., and Ciccarelli, A. S. 1970. Another type of *Clostridium botulinum*. Zentralblatt fur Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, Abt. I (Originale), 215(2), 221-4.

Grave, K., Kaldhusdal, M., Kruse, H., Harr, L.M.F. and Flatlandsmo, K. 2004. What has happened in Norway after the ban of avoparcin? Consumption of antimicrobials by poultry. Preventive Veterinary Medicine, 62(1), 59-72.

Grimont, P. A., and Weill, F. X. 2007. Antigenic formulae of the *Salmonella* serovars. WHO collaborating centre for reference and research on *Salmonella*, 9, 1-166.

Hacking, W. C., Mitchell, W. R., and Carlson, H. C. 1978. *Salmonella* investigation in an Ontario feed mill. Canadian Journal of Comparative Medicine, 42(4), 400.

Hafez, H. M. 2011. Enteric diseases of poultry with special attention to *Clostridium perfringens*. Pakistan Veterinary Journal, 31(3), 175-184.

Herikstad, H., Motarjemi, Y., and Tauxe, R. V. 2002. *Salmonella* surveillance: a global survey of public health serotyping. Epidemiology & Infection, 129(1), 1-8.

Hofacre, C. L., Froyman, R., Gautrias, B., George, B., Goodwin, M. A., and Brown, J. 1998. Use of Aviguard and other intestinal bioproducts in experimental *Clostridium perfringens*-associated necrotizing enteritis in broiler chickens. Avian Diseases, 42(3), 579-584.

Hoffmann, S., Maculloch, B., and Batz, M. 2015. Economic burden of major foodborne illnesses acquired in the United States. United States Department of Agriculture, Economic Research Service.

International Feed Industry Federation (IFIF), 2020. The global feed industry. Accessed March 2020. Available at https://ifif.org/global-feed/industry/

Ispolatovskaya, M. V. 2012. Type A *Clostridium perfringens* toxin. Microbial toxins, 2, 109-58.

Izat, A.L., Colberg, M., Reiber, M.A., Adams, M.H., Skinner, J.T., Cabel, M.C., Stilborn, H.L. and Waldroup, P.W. 1990. Effects of different antibiotics on performance, processing characteristics, and parts yield of broiler chickens. Poultry Science, 69(10), 1787-1791.

Jayas, D. S., and White, N. D. 2003. Storage and drying of grain in Canada: low cost approaches. Food Control, 14(4), 255-261.

Jayas, D. S., White, N. D., and Muir, W. E. (Eds.). 1994. Stored-grain ecosystems (Vol. 39). CRC Press.

Jean, D., Briolat, V., and Reysset, G. 2004. Oxidative stress response in *Clostridium perfringens*. Microbiology, 150(6), 1649-1659.

Jordan, F. 2001. Clostridia. Pages 154-162 in Poultry Diseases. Jordan, F., Pattison, M., Alexander, D., and Faragher, T., 5th edition. WB Saunders Publishers.

Kaper, J. B., Nataro, J. P., and Mobley, H. L. 2004. Pathogenic *Escherichia coli*. Nature Reviews Microbiology, 2(2), 123-140.

Knarreborg, A., Simon, M. A., Engberg, R. M., Jensen, B. B., and Tannock, G. W. 2002. Effects of dietary fat source and subtherapeutic levels of antibiotic on the bacterial community in the ileum of broiler chickens at various ages. Applied and Environmental Microbiology, 68(12), 5918-5924.

Keyburn, A.L., Boyce, J.D., Vaz, P., Bannam, T.L., Ford, M.E., Parker, D., Di Rubbo, A., Rood, J.I. and Moore, R.J. 2008. *netB*, a new toxin that is associated with avian necrotic enteritis caused by *Clostridium perfringens*. PLOS Pathogens, 4(2): e26.

Keyburn, A. L., Yan, X. X., Bannam, T. L., van Immerseel, F., Rood, J. I., and Moore, R. J. 2010. Association between avian necrotic enteritis and *Clostridium perfringens* strains expressing NetB toxin. Veterinary Research, 41(2), 1-8.

Kidd, R. S., Rossignol, A. M., and Gamroth, M. J. 2002. *Salmonella* and other Enterobacteriaceae in dairy-cow feed ingredients: antimicrobial resistance in western Oregon. Journal of Environmental Health, 64(9), pp. 9.

Kokić, B., Lević, J., Chrenková, M., Formelová, Z., Poláčiková, M., Rajský, M., and Jovanović, R. 2013. Influence of thermal treatments on starch gelatinization and in vitro organic matter digestibility of corn. Food and Feed research, 40(2), 93-99.

Leeson, S., Caston, L., Kiaei, M. M., and Jones, R. 2000. Commercial enzymes and their influence on broilers fed wheat or barley. Journal of Applied Poultry Research, 9(2), 242-251.

Lück, E., and Jager, M. 2013. Chemische Lebensmittelkonservierung: Stoffe—Wirkungen—Methoden. Springer-Verlag.

M'Sadeq, S. A., Wu, S. B., Choct, M., and Swick, R. A. 2015. Heat production and energy efficiency of broilers infected with necrotic enteritis. Avian diseases, 60(1), 50-55.

Maciorowski, K. G., Herrera, P., Jones, F. T., Pillai, S. D., and Ricke, S. C. 2007. Effects on poultry and livestock of feed contamination with bacteria and fungi. Animal Feed Science and Technology, 133(1-2), 109-136.

Maciorowski, K. G., Herrera, P., Kundinger, M. M., and Ricke, S. C. 2006. Animal feed production and contamination by foodborne *Salmonella*. Journal für Verbraucherschutz und Lebensmittelsicherheit, 1(3), 197-209.

McClane, B. A. 1996. An overview of *Clostridium perfringens* enterotoxin. Toxicon, 34(11-12), 1335-1343.

Niilo, L. 1980. *Clostridium perfringens* in animal disease: a review of current knowledge. The Canadian Veterinary Journal, 21(5), 141.

Nurmi, E., and Rantala, M. 1973. New aspects of *Salmonella* infection in broiler production. Nature, 241(5386), 210-211.

Petit, L., Gibert, M., and Popoff, M. R. 1999. *Clostridium perfringens*: toxinotype and genotype. Trends in Microbiology, 7(3), 104-110.

Popoff, M. R. 2016. Toxins of histotoxic clostridia: *Clostridium chauvoei, Clostridium septicum, Clostridium novyi*, and *Clostridium sordellii*. Clostridial Diseases of Animals, 23.

Popoff, M. R., and Stiles, B. G. 2005. 17 Clostridial toxins vs. other bacterial toxins. Handbook on clostridia, 323.

Prió, P., Gasol, R., Soriano, R. C., and Perez-Rigau, A. 2001. Effect of raw material microbial contamination over microbiological profile of ground and pelleted feeds. Brufan Journal, 197-199.

Public Health Agency of Canada. 2011. Pathogen Safety Data Sheets: Infectious Substances: *Clostridium perfringens*. Accessed June 2019. Available at https://www.canada.ca/en/public-health/services/laboratory-biosafety-biosecurity/pathogen-safety-data-sheets-risk-assessment/clostridium.html

Revitt-Mills, S.A., Rood, J.I. and Adams, V. 2015. *Clostridium perfringens* extracellular toxins and enzymes: 20 and counting. Microbiology Australia, 36(3), 114-117.

Rood, J. I. 1998. Virulence genes of *Clostridium perfringens*. Annual review of Microbiology, 52(1), 333-360.

Rood, J.I., Adams, V., Lacey, J., Lyras, D., McClane, B.A., Melville, S.B., Moore, R.J., Popoff, M.R., Sarker, M.R., Songer, J.G. and Uzal, F.A. 2018. Expansion of the *Clostridium perfringens* toxin-based typing scheme. Anaerobe, 53, 5-10.

Roth, F. X., and Kirchgessner, M. 1998. Organic acids as feed additives for young pigs: Nutritional and gastrointestinal effects. Journal of Animal Feed Sciences, 7(Suppl 1), 25-33.

Russell, J.B. and Jarvis, G.N. 2001. Practical mechanisms for interrupting the oral-fecal lifecycle of *Escherichia coli*. Journal of Molecular Microbiology and Biotechnology, 3(2), 265-272.

Sapkota, A. R., Lefferts, L. Y., McKenzie, S., and Walker, P. 2007. What do we feed to food-production animals? A review of animal feed ingredients and their potential impacts on human health. Environmental Health Perspectives, 115(5), 663-670.

Schwarz, S., Kehrenberg, C., and Walsh, T. R. 2001. Use of antimicrobial agents in veterinary medicine and food animal production. International journal of Antimicrobial Agents, 17(6), 431-437.

Shimizu, T., Ohtani, K., Hirakawa, H., Ohshima, K., Yamashita, A., Shiba, T., Ogasawara, N., Hattori, M., Kuhara, S. and Hayashi, H. 2002. Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. Proceedings of the National Academy of Sciences, 99(2), 996-1001.

Silva, R. O., Uzal, F. A., Oliveira, C. A., and Lobato, F. C. 2016. Gas gangrene (malignant edema). Clostridial diseases of animals. Ames: Wiley Blackwell, 243-54.

Singh, V. 2013. *Salmonella* serovars and their host specificity. Journal of Veterinary Science & Animal Husbandry, 1(301), 10-15744.

Singh, B. M. 2017. The Effects of Feeding Reduced-oil DDGS to Broilers when Challenged with *C. perfringens* and *Eimeria* spp. on Necrotic Enteritis and Intestinal Microbiome. Electronic Theses and Dissertations. Auburn University.

Skinner, J. T., Bauer, S., Young, V., Pauling, G., and Wilson, J. 2010. An economic analysis of the impact of subclinical (mild) necrotic enteritis in broiler chickens. Avian Diseases, 54(4), 1237-1240.

Skřivanová, E., Marounek, M., Benda, V., and Březina, P. 2006. Susceptibility of *Escherichia coli*, *Salmonella* sp and *Clostridium perfringens* to organic acids and monolaurin. Veterinární Medicína, 51(3), 81-88.

Sonnabend, O. A., Sonnabend, W., Heinzle, R., Sigrist, T., Dirnhofer, R., and Krech, U. 1981. Isolation of *Clostridium botulinum* type G and identification of type G botulinal toxin in humans: report of five sudden unexpected deaths. Journal of Infectious Diseases, 143(1), 22-27.

Suen, J. C., Hatheway, C. L., Steigerwalt, A. G., and Brenner, D. J. 1988. *Clostridium argentinense* sp. nov.: a genetically homogeneous group composed of all strains of *Clostridium botulinum* toxin type G and some nontoxigenic strains previously identified as *Clostridium subterminale* or *Clostridium hastiforme*. International Journal of Systematic and Evolutionary Microbiology, 38(4), 375-381.

Tessari, E. N. C., Cardoso, A. L. S. P., Kanashiro, A. M. I., Stoppa, G. F. Z., Luciano, R. L., and de Castro, A. G. M. 2014. Analysis of the presence of *Clostridium perfringens* in feed and raw material used in poultry production. Food and Nutrition Sciences, 2014.

Titball, R. W., Naylor, C. E., and Basak, A. K. 1999. The *Clostridium perfringens* α-toxin. Anaerobe, 5(2), 51-64.

Tuite, J., and Foster, G. H. 1979. Control of storage diseases of grain. Annual Review of Phytopathology, 17(1), 343-366.

Udhayavel, S., Ramasamy, G. T., Gowthaman, V., Malmarugan, S., and Senthilvel, K. 2017. Occurrence of *Clostridium perfringens* contamination in poultry feed ingredients: Isolation, identification and its antibiotic sensitivity pattern. Animal Nutrition, 3(3), 309-312.

van Immerseel, F., Buck, J. D., Pasmans, F., Huyghebaert, G., Haesebrouck, F., and Ducatelle, R. 2004. *Clostridium perfringens* in poultry: an emerging threat for animal and public health. Avian pathology, 33(6), 537-549.

van Immerseel, F., Rood, J. I., Moore, R. J., and Titball, R. W. 2009. Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens. Trends in Microbiology, 17(1), 32-36.

Wade, B., and Keyburn, A. 2015. The true cost of necrotic enteritis. Poultry World. Accessed January 2020. Available at https://www.poultryworld.net/Meat/Articles/2015/10/The-true-cost-of-necrotic-enteritis-2699819W/

Walker, J. H. C. 1959. *Salmonella* Organisms in Animal Feeding Stuffs and Fertilizers. Monthly Bull. Ministry of Health & Pub. Health Lab. Service (directed by Med. Res. Council), 18, 26-35.

White, N. D. 1995. Insects, mites and insecticides in stored-grain ecosystems. Pages 123-168 in Stored grain ecosystems. Jayas D., White N. and Muir W. Marcel Dekker, Inc.

Williams, R. B. 2002. Anticoccidial vaccines for broiler chickens: pathways to success. Avian Pathology, 31(4), 317-353.

Williams, R. B., Marshall, R. N., La Ragione, R. M., and Catchpole, J. 2003. A new method for the experimental production of necrotic enteritis and its use for studies on the relationships between necrotic enteritis, coccidiosis and anticoccidial vaccination of chickens. Parasitology Research, 90(1), 19-26.

Wojdat, E., Kwiatek, K., and Kozak, M. 2006. Occurrence and characterization of some *Clostridium* species isolated from animal feedingstuffs. Bulletin-Veterinary Institute in Pulawy, 50(1), 63.

Yegani, M., and Korver, D. R. 2008. Factors affecting intestinal health in poultry. Poultry Science, 87(10), 2052-2063.

Zang, J. J., Piao, X. S., Huang, D. S., Wang, J. J., Ma, X., and Ma, Y. X. 2009. Effects of feed particle size and feed form on growth performance, nutrient metabolizability and intestinal morphology in broiler chickens. Asian-Australasian Journal of Animal Sciences, 22(1), 107-112.

Zhang, L., Zhang, L., Zeng, X., Zhou, L., Cao, G., and Yang, C. 2016. Effects of dietary supplementation of probiotic, *Clostridium butyricum*, on growth performance, immune response, intestinal barrier function, and digestive enzyme activity in broiler chickens challenged with Escherichia coli K88. Journal of Animal Science and Biotechnology, 7(1), 3.

Zhao, X., Yang, J., Wang, L., Lin, H., and Sun, S. 2017. Protection mechanism of *Clostridium butyricum* against *Salmonella Enteritidis* infection in broilers. Frontiers in Microbiology, 8, 1523.

Table 2.1. Some possible causes of enteric disorders in poultry.

Non-Infectious	Infectious
Feed	**Viral agents**
Ingredient quality	Reovirus, Astrovirus, Enterovirus,
Palatability	Rotavirus, Coronavirus enteritis
Formulation	Influenza A
Pellet quality	**Bacterial agents**
Management	*Salmonella*
Available feed space	*E. coli*
Available water space	Clostridia
Distribution of feeders	**Mycotic Agents**
Distributions of waterers	Candida
Air quality	**Parasites**
Temperature	Coccidia
Stocking density	Histomonas
Litter quality	Hexaminta, Ascaridia

Source: Hafez (2011)

Table 2.2. Partial list of feed ingredients for animal feeds legally used in the US[1].

Origin	Examples
Plant	
Grains	Barley, corn, oats, rice sorghum and wheat
Plant protein products	Canola meal, cottonseed cakes and meals, peanut meal, safflower meal, and soybean feed and meal
Processed grain by products	Distillers products, brewers dried grains, corn gluten, sorghum germ cake and meal, peanut skins, and wheat bran
Molasses	Beet, citrus, starch, and cane molasses
Miscellaneous	Almond hulls and ground shells, buckwheat hulls, legumes and their by-products, and other crop by-products
Animal	
Rendered animal protein	Meat meal, meat meal tankage, meat and bone meal, poultry meal, animal by product meal, dried animal blood, blood meal, feather meal.
Animal waste	Dried ruminant waste, dried swine waste, dried poultry litter, and undried processed animal waste products
Marine by-products	Fish meal, fish residue meal, crab meal, shrimp meal, fish oil, fish liver and glandular meal, and fish by-products
Dairy products	Dried cow milk, casein, whey products, and dried cheese
Mixed	
Fats and Oils	Animal fat, vegetable fat and hydrolyzed fats
Restaurant food waste	Edible food waste from restaurants, bakeries and cafeterias
Other	
Antibiotics	Tetracyclines, macrolides, fluoroquinolones, and streptogramins
Other metal compounds	Copper compounds and metal amino acid complexes
Nonprotein nitrogen	Urea, ammonium chloride, and ammonium sulfate
Minerals	Bone charcoal, calcium carbonate, chalk rock, iron salts, magnesium salts, and oyster shell flour
Vitamins	Vitamins A, D, B12, E, niacin, and betaine
Enzymes	Phytase, cellulase, lactase, lipase, pepsin, and catalase

[1] Data adapted from Sapkota (2007).

Chapter 3.0 Evaluation of Commercially Manufactured Animal Feeds to Determine Presence of *Salmonella*, *E. coli* and *Clostridium perfringens*

Introduction:

Clostridium perfringens (CP) is one of the leading foodborne pathogens causing disease and illness in humans. Just in the United States, the Center for Disease Control and Prevention (CDC) estimates that CP causes nearly 1 million cases of foodborne illness every year with 438 hospitalizations and 26 deaths (ERS, 2014; CDC, 2018). Additionally, the infection with CP has a large economic impact with an estimated financial loss of 343 million dollars due to medical costs, productivity loss and premature deaths (ERS, 2014).

CP is an organism that is found worldwide and in animal live productions is responsible for causing diseases such as necrotic enteritis (NE). In the poultry industry the financial burden of this disease was estimated to be between 5–6 billion dollars/year (Wade and Keyburn, 2015). NE is characterized by necrosis and inflammation in the chicken's intestinal tract, reducing the ability of the bird to absorb nutrients; therefore, directly impacting the growth performance, feed conversion ratio and profitability of the operation (Wade and Keyburn, 2015). Historically, NE has been controlled by adding antibiotic growth promoters (AGPs) into the chicken's feed (van Immerseel et al., 2009). However, due to the risk of spreading antibiotic resistance, the poultry industry as a whole is moving away from this practice, which has led to an increase in NE incidence (van Immerseel et al., 2009). The best way to reduce exposure of the birds to this pathogen is by addressing the risk from different sources, these include providing good broiler house environment management—i.e. clean, pathogen free litter, good temperature and humidity control—, ensuring biosecurity practices at all times—i.e. providing clean, pathogen free sources of feed and water—

, keeping the flocks up to date with the vaccination programs and providing the correct diets to avoid colonization of the bird's intestinal tract by pathogens.

Contaminated animal feeds expose the animals to pathogens which may lead to infection or colonization of the gastrointestinal tract (Crump et al., 2002). The contamination of the feed with bacterial pathogens may occur at the harvest/transportation of the feed ingredients, processing at the feed mill or at any point during storage or transportation to the farm (Maciorowski et al., 2006). These bacteria have the potential to colonize the animal leading to disease of the animal and/or during processing may lead to contamination of the carcasses with foodborne pathogens (Crump et al., 2002). There is substantial evidence that animal feeds are often contaminated with bacteria such as *Salmonella* (Walker, 1959; Hacking et al., 1978; Kidd et al., 2002; Maciorowski et al., 2006), *E. coli* (Davis et al., 2003; Dargatz et al., 2005) and CP (Wojdat et al., 2006; Tessari et al., 2014; Udhayavel et al., 2017). These studies show that contamination of the animal feeds is related to the addition of a contaminated ingredient such as protein meals into the mixing process at the feed mill. Cereal grains also represent a source of potential contamination to the animal feeds due to the diverse microbial population that can be present on them. Some of the bacteria found include Clostridial spp. (*C. perfringens* and *C. botulinum*), *E. coli* and *Salmonella* (Maciorowski et al., 2007). Microbiological evaluation of the ingredients and finished feeds becomes a key element to ensure that the feed is not a source of contamination and at the same time this evaluation could also be used as an indicator of quality assurance and feed safety during its production until feeding the animals at the farm (Wojdat et al., 2005). Further investigation is warranted to determine if animal feeds are a contamination source to farm animals. Therefore, the objective of this study was to establish if commercially manufactured animal feeds or feed ingredients used in

its production serve as a source of contamination of *Salmonella*, *E. coli* and/or *Clostridium perfringens*.

Materials and Methods:

Sample Collection:

Table 3.1 describes the feed mills involved in this study. From the five feed mills, four were commercial feed mills, with production rates between 1,500 – 24,000 tons/month, and the other one was the research feed mill at Auburn University. A total of 292 samples (132 of feed ingredients and 160 of mixed feeds) were collected during two sampling periods (Nov-Mar and Apr-Oct) from 5 different locations within each feed mill: ingredient receiving, post mixing, post pelleting (hot sample), post cooling, and at loadout. A 3.175 cm PVC pipe with a 3.175 cm cap socket at one end was used to collect the samples. On the end that the pipe did not have the socket, the pipe was cut into the side, creating a window that facilitated the collection of the samples. The PVC pipe was sanitized with 70% ethanol between every sample and it was allowed to dry before the collection of the following sample. The samples were directly collected from the trucks/trains delivering ingredients (with the exception of corn meal which was collected in the plant after being milled) or at their respective stage of production during processing as previously described. A total of four samples were collected at each sampling point at intervals of 6-8 minutes. Approximately 100 grams of sample were collected and directly placed into a sterile Whirl-Pak™ bag (Nasco®) and placed in a cooler with icepacks to be transported from the feed mill to the laboratory at Auburn University. All samples were refrigerated (4°C) upon arrival until the microbiological analysis was performed.

Microbiological Analysis:

Samples of feed and feed ingredients were processed by weighing 10 grams of the sample and putting them into sterile filter Whirl-Pak™ bag (Nasco®) to which 90 ml of Phosphate Buffered Saline (PBS; VWR, VWR Chemicals, Fountain Parkway, Solon, Ohio, USA) was added. Each bag was then stomached for 60 seconds and then serially diluted using PBS. The serial dilutions (1:10) were performed by adding 100 µL of previous dilution into tubes containing 900 µL of PBS until the desired dilutions were achieved. From the dilution tubes, 100 µL were spread plated or 10 µL were spot dropped in triplicate onto selective media plates. Each dilution of every sample was spread plated onto Tryptose Sulfite Cycloserine agar (TSC, Merck KGaA, EMD Millipore Corporation, Germany) for *Clostridium perfringens* isolation and spot dropped onto Xylose Lysine Tergitol 4 Agar (XLT4; Criterion™, Hardy Diagnostics, Santa Maria, CA, USA) and MacConkey agar (BD, Becton, Dickinson and Company, Sparks, MD, USA) for *Salmonella* and *E. coli* isolation, respectively. The TSC plates were incubated in an anaerobic chamber (Bactron IV™ Anaerobic/ Environmental chamber, Shel Lab, Cornelius, OR, USA) containing 5% CO_2, 5% H_2 90% N_2 at 40°C for 48 hours. All the other plates were incubated at 37°C aerobically for 24 hours. After the incubation period, all of the plates were removed from the incubators and the individual indicative colonies for each organism were then enumerated—i.e. black colonies on TSC as indicative of *Clostridium perfringens*. A portion of the black colonies (4 per sample when possible) on the TSC were isolated and cultured onto pre-reduced Tryptic Soy Agar II plates containing 5% Sheep Blood (TSA + 5% SB; BD BBL™, Becton, Dickinson, and Company, Sparks, MD, USA). They were anaerobically incubated for 24-36 hours at 40°C. The isolates that displayed double zone beta-hemolysis—a distinguishing characteristic of *Clostridium perfringens* from other Clostridial spp. (Tille, 2015)—were then cultured in Brain Heart Infusion Broth (BHIB; Criterion™, Hardy Diagnostics, Santa Maria, CA, USA) with 20% glycerol, and

frozen in cryovials with sterile beads at -80°C. This was performed so that they could later be confirmed as *Clostridium perfringens* by PCR and biochemical tests. When these isolates were further assayed, each isolate was grown by placing one or two beads from the cryovial onto a pre-reduced TSA + 5% SB plate which was then incubated in the anaerobic chamber at 40°C for 24 hours. The assays performed on these cultures were gram stain reaction, lecithinase test, spore forming test and PCR.

Based on previous trials not included in this research, it was identified that not all feed or feed ingredients samples had bacterial growth when plated onto the selective media used for this study. Therefore, during the same day that the samples were plated onto the selective media, one mL of the 10:1 dilution from each sample was added to five mL of enrichment media and incubated for 48-72 hours under the same conditions used for the selective plating. For *Clostridium perfringens* and *E. coli* the enrichment media used was BHIB and for *Salmonella* enrichment Tetrathionate Brilliant Green Broth tubes (TTB, HiMedia®, HiMedia Laboratories Pvt. Ltd, Mumbai, India) were used. The enriched cultures were then streaked onto TSC, XLT4 and MacConkey agar plates using 1 µL disposable loops (VWR International, LLC, Radnor, PA) and incubated for 48 hours under the same conditions as before to detect if *Clostridium perfringens*, *Salmonella* or *E. coli* were present in the original samples. For those samples that were positive after enrichment but did not show any countable colonies in the spread plating/spot dropping, a value of 10 colony-forming unit was assigned.

DNA Extraction:

Each presumptive *Clostridium perfringens* isolate was grown by placing a single bead from the cryovial onto pre-reduced TSA+5% SB plate, which was incubated in the anaerobic chamber at 40°C for 24 hours. A single colony exhibiting either double-zone beta hemolysis or alpha

hemolysis was chosen and transferred into 5 mL of BHIB and incubated in the anaerobic chamber at 40°C for 24 hours. The bacterial DNA from each isolate was extracted using the Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA) according to the manufacturer instructions. The extracted DNA was tested for concentration and purity using 1.5 µL of each DNA extraction using a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, Bremen, Germany) according to manufacturer instructions. The standard for purity was a 260nm/280nm ratio between 1.8 - 2.0. The extracted DNA was stored at 4°C until further analysis.

Clostridium perfringens Detection by Polymerase Chain Reaction (PCR):

All the obtained presumptive *Clostridium perfringens* isolates were assayed for genetic analysis by PCR. The primers used in this study were targeted to detect the alpha toxin (*cpa*) and Necrotic Enteritis Toxin B-like (*netB*) genes. The primer sequences used in this study were previously published by Bailey et al. (2013) (*cpa* F -GCAGCAAAGGTAACTCTAGCTAACT- *cpa* R -CCTGGGTTGTCCATTTCCCATT-) and by Keyburn et al. (2010) (*netB*5 F -CGCTTCACATAAAGGTTGGAAGGC- *netB*5 R -TCCAGCACCAGCAGTTTTTCCT-). All samples were processed with the same reagent concentrations for each reaction and it consisted of the following: 12.5 µL of EconoTaq® PLUS 2X Master Mix (Lucigen, Middleton, WI, USA), 0.25 µL of each forward and reverse *cpa*/*netB* primers (Eurofins Genomics LLC, Louisville, KY, USA) at a concentration of 100 µM, 3 µL of extracted DNA and 9 µL of RNAse/DNAse-free water (25 µL total per reaction). The DNA amplification was performed using an iQ^{TM}5 thermocycler (Bio-Rad, Hercules, CA, USA). The PCR cycle parameters for the *cpa* detection were as follows: initial denaturing at 95°C for 5 minutes, 15 touchdown cycles at 94°C for 30 seconds (denaturing), 65°C for 30 seconds (decreasing by 1°C every 1 cycle for annealing) and 72°C for 30 seconds

(extension), followed by 25 cycles at 94°C for 30 seconds (denaturing), 50°C for 30 seconds (annealing) and 72°C for 30 seconds (extension) and a final elongation at 72°C for six minutes. The PCR cycle for *netB* detection did not have the touchdown portion, instead after the initial denaturing they were run for 40 cycles at 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. The final elongation remained as in the *cpa* parameters and in both cases after the final elongation, the reactions were held at 4°C until they were removed from the thermocycler.

The PCR products were separated by gel electrophoresis using a 2% agarose gel (IBI Scientific, Dubuque, IA, USA). The gels and buffer used during analysis were made from 1X Tris-Acetate-EDTA (TAE) buffer (Bio-Rad, Hercules, CA, USA). Prior to the addition of the PCR products (10 µL per sample) into the wells in the gel, they were individually mixed with a solution formed by 2 µL 6X bromophenol blue (loading buffer) and 1 µL of SYBR® Green Nucleic Acid Gel Stain (Lonza, Rockland, ME, USA) to allow the PCR product bands to be observed in the gel (230 base-pairs for *cpa* and 316 base-pairs for *netB*). The gel was then run at 90v for 1 hour to allow the separation of the PCR products. A 100 bp DNA molecular weight marker (VWR Chemicals, LLC, Solon, OH, USA) was used as the size standard in the gel. A negative control (deionized, distilled water) and a positive control (*C. perfringens* isolates previously confirmed as *cpa*/*netB* positive) were used during each gel electrophoresis run. After the run, the gel was placed onto a c200 gel imaging workstation (Azure Biosystems, Dublin, CA, USA) to capture the gel image and view the separated bands that confirmed the presence or not of the genes of interest.

Data Analysis:

All statistical analysis were conducted using IBM® SPSS® software version 26. Data containing the $\log_{10}$ transformation of colony counts per media type per sample were used to compare differences in incidences in the ingredient samples based on the feed mills and to compare

differences in incidences among the production process samples of each feed mill. All data was analyzed using a General Linear Model (GLM). Significant differences were reported at $P \leq 0.05$ and means were separated using Tukey's HSD.

Results:

Before presenting the results found in this study it's important to emphasize that due to the number of samples collected, and the limited number of feed mills assayed, the data presented is only representative to the actual feed ingredients and feed loads that were sampled. Nevertheless, the data presented in this study may be used as reference to estimate the levels of contamination on feed ingredients and animal feeds that are produced at commercial or integrated feed mills. Additionally, from all the samples collected and processed in this study no *Salmonella* was recovered on selective media or enrichments, therefore no $\log_{10}$ from the colony-forming unit (CFU)/gram were included in the data presented.

Table 3.2 summarizes the mean $\log_{10}$ values for *Clostridium* spp. counts (CSC) and *E. coli* counts (ECC) obtained from the feed ingredients and feed samples collected across the five feed mills sampled. The ingredient samples collected showed significant ($P < 0.05$) difference in both CSC and ECC. Peanut meal and corn gluten meal were the ingredients with the highest contamination levels for CSC (3.91 $\log_{10}$ and 2.61 $\log_{10}$, respectively) while peanut meal and corn meal were the ingredients with highest ECC (4.15 $\log_{10}$ and 2.85 $\log_{10}$, respectively). Soybean meal (CSC 0.42 $\log_{10}$, ECC 0.49 $\log_{10}$) and distillers dried grains with solubles (DDGS) (CSC 0.20 $\log_{10}$, ECC 0.92 $\log_{10}$) were the ingredients displaying the lowest levels of contamination for both CSC and ECC. For the samples collected during the feed manufacturing process, no statistical difference was observed in the means of CSC, but a higher contamination level was observed at

the post mixing stage for ECC (3.03 $\log_{10}$) when compared to the rest of the manufacturing process samples.

The data shown in the table 3.3 shows the effect of sampling period on CSC and ECC obtained from the ingredient samples collected throughout the study. Sampling period did not have a relatively consistent effect on CSC when the ingredient samples by feed mill were compared, the exceptions were corn meal (feed mill E) and soybean meal (feed mill C). For these two ingredients the CSC were higher ($P < 0.05$) on the samples collected between Apr-Oct—corn meal (3.44), soybean meal (2.48)—than the samples collected between Nov-Mar—corn meal (2.22), soybean meal (no colony growth from any sample). For ECC the ingredients DDGS (feed mill A) and soybean meal (feed mill C) were statistically different when compared between sampling periods ($P < 0.05$). DDGS had higher ECC on the samples collected from feed mill A between Nov-Mar (3.61) than the samples collected between Apr-Oct (no colony growth from any sample) and soybean meal had higher ECC on those samples collected from feed mill C between Apr-Oct (2.18) than the ones collected between Nov-Mar (0.50).

Table 3.3 also allows us to compare the bacterial contamination of each ingredient among the different feed mills sampled. For the samples collected between Nov-Mar the ingredients corn meal and soybean meal had different levels of contamination for CSC and ECC, respectively ($P < 0.05$). The feed mill displaying the highest CSC on corn meal was feed mill C (3.08), whereas feed mills A displayed the lowest contamination level (0.25). For ECC, soybean meal was statistically higher at feed mill A (1.00) than the rest of the feed mills with the exception of feed mill C (0.50). For the samples collected between Apr-Oct the ingredients corn meal, soybean meal and DDGS had different levels of contamination on CSC and soybean meal was the only ingredient showing difference with ECC ($P < 0.05$). For corn meal, the CSC were higher in feed mill E (3.44) being

statistically different than feed mills A (1.37) and feed mill D (1.08). For soybean meal, the CSC were highest in feed mill C (2.48) being statistically different than the rest of the feed mills; feed mills A and D had the lowest contamination levels (no colony growth from collected samples). For DDGS, the CSC were higher in feed mill E (0.75) than in feed mills A and B (no colony growth from collected samples). For the ECC, soybean meal from feed mill C (2.18) was statistically different than feed mills A, D and E (no colony growth from collected samples). It is interesting to note that the corn meal samples collected across the five feed mills during the two sampling periods did not have any statistical difference in ECC, indicating that the loads of corn meal sampled were equally contaminated with *E. coli* among the five feed mills at both sampling dates.

The effect of sampling periods on CSC and ECC from the feed samples obtained during the different stages of feed processing at each feed mill are shown in table 3.4. When compared between sampling periods, samples taken from feed mill A (post mixing) and feed mill D (post mixing, post pelleting and post cooling) showed a significant difference ($P < 0.05$) in CSC and the samples taken at feed mill B (post pelleting) showed statistical difference in ECC. In feed mill A, the post mixer samples collected between Apr-Oct had higher CSC (2.15) than those collected at the same point between Nov-Mar (1.00) ($P < 0.05$). In feed mill D, the samples collected at post mixing, post pelleting and post cooling had higher CSC in the samples collected between Nov-Mar (2.43, 2.12 and 3.22, respectively) than those collected at the same points between Apr-Oct (0.50, 0.25 and 0.25, respectively). In feed mill B, the post pelleting samples collected between Apr-Oct (1.58) had higher ECC ($P < 0.05$) than those samples collected at the same point between Nov-Mar (0.25).

Table 3.4 also allow us to compare the bacterial contamination at each stage of the production process among the different feed mills sampled. All the process samples collected in

both sampling periods showed different CSC, therefore indicating different levels of contamination among feed mills at a particular sampling point with the exception of those collected at post pelleting and loadout between Nov-Mar ($P < 0.05$). For CSC in general, those samples collected from feed mills B and E were more contaminated than the samples collected at the same stages of processing of other feed mills ($P < 0.05$). That holds true for samples collected in both sampling periods, with the exceptions of the post pelleting and loadout stages of processing samples collected between Nov-Mar. For ECC, the post mixing (Nov-Mar) and post pelleting (Apr-Oct) samples were the ones that had difference among the contamination in the feed mills ($P < 0.05$). On the post mixing samples (Nov-Mar), feed mill C (1.65) showed the lowest ($P < 0.05$) levels of contamination with *E. coli*, and on the post pelleting samples (Apr-Oct), feed mill B (1.58) was the one that showed the highest ($P < 0.05$) levels of contamination. It's important to point out that feed mills A and B were the only ones on which *E. coli* was recovered after the pelleting process.

Figures 3.1-3.5 illustrate the interaction between the feed samples collected at every stage of processing by each feed mill (A-E). In general, a relatively consistent pattern was observed in all feed mills sampled, this pattern indicates that significant differences were noticed in the ECC, but no statistical differences were observed in the CSC using GLM ($P < 0.05$). Figure 3.1 demonstrates that for feed mill A the bacterial contamination with Clostridial spp. was not any different between the stages of processing samples collected (including mash and pellet samples) during both sampling periods ($P > 0.05$). At the same time, this figure shows the significant reduction in *E. coli* contamination after submitting the mash feed (post mixer samples) to the pelleting process ($P < 0.05$). For samples collected between Nov-Mar, a complete reduction of *E. coli* was observed after pelleting the feed, and no *E. coli* colonies were detected throughout the

rest of the manufacturing process. For samples collected between Apr-Oct, even though a significant reduction was achieved in ECC ($P < 0.05$), the bacterium *E. coli* was recovered from the post pelleting (0.50) and the loadout (0.25) samples.

Figure 3.2 shows the Clostridial spp. and *E. coli* contamination over the feed manufacturing process at feed mill B. It can be observed that the contamination with Clostridial spp. was relatively stable (the samples remained on the same $\log_{10}$ value as feed samples were collected in subsequent processing stages) and that no differences were observed between the samples collected over different sampling periods ($P > 0.05$). For ECC, figure 3.2 displays a significant difference between the post mixing stage and the rest of the process samples collected during both sampling periods ($P < 0.05$). It is interesting to note that during both sampling periods, *E. coli* was recovered in samples collected after the pelleting process. For the samples collected between Nov-Mar, even though that a significant reduction ($P < 0.05$) in ECC existed between the post mixing samples (4.84), the bacterium *E. coli* still was recovered from the post pelleting (0.25) and the loadout (0.25) samples. For the samples collected between Apr-Oct, the mash samples collected at the post mixing stage (3.36) were not significantly different ($P > 0.05$) in the bacterial contamination with *E. coli* from those samples collected post pelleting (1.58). A significant reduction ($P < 0.05$) was observed until the post cooling (0.75) and loadout (0.50) stages.

In the figure 3.3 the contamination of Clostridial spp. and *E. coli* from the samples obtained in the production process of feed mill C are presented. The bacterial contamination with Clostridial spp. remained at or under 1.00 with the exception of the loadout sample collected between Apr-Oct, which was 1.62, the CSC were not significantly different ($P > 0.05$) which means that the contamination with these bacteria was stable through the production process and the same between sampling periods. For the ECC, we can observe on figure 3.3 that when the feed passed the

pelleting stage, no *E. coli* was recovered from any further sample (both sampling periods). The ECC were significantly higher in the post mixing samples collected between Apr-Oct (4.04) compared to the rest of the samples collected in the same sampling period ($P < 0.05$).

Figure 3.4 displays the recovery of Clostridial spp. and *E. coli* from the samples obtained in the production process of feed mill D. No difference in the means of Clostridial spp. were noted in the samples collected from the production process during the two sampling periods. This means that the CSC were not different from one stage of processing to another on both times the feed mill was sampled. A significant reduction of the mean CSC at each stage of processing was observed when compared between sampling periods (refer to table 3.4) but not when the samples were compared by the manufacturing process. For the ECC, the same pattern was observed during both sampling periods. The ECC from the post mixing samples from Nov-Mar (2.05) and from Apr-Oct (3.42) were statistically higher than the rest of the stages of processing for each time the feed mill was sampled. For the remaining processing samples (post pelleting, post cooling and loadout) no bacterial growth was observed from the samples collected. This indicates that the pelleting process in this feed mill was effective both times that samples were collected because no *E. coli* was recovered after the feed passed through the conditioning temperatures prior to pelleting.

Figure 3.5 shows the bacterial contamination with Clostridial spp. and *E. coli* for the samples collected at feed mill E. It can be observed that bacterial contamination with Clostridial spp. was relatively stable for both sampling periods, no major fluctuations over the CSC were observed therefore no statistical significance was observed between the stages of processing ($P > 0.05$). For the incidence of *E. coli*, it was observed that the samples collected at the post mixing stage of processing on both Nov-Mar (2.77) and Apr-Oct (2.61) sampling periods were the only

samples from the whole manufacturing process that had ECC. This finding being statistically different than the rest of the stages of processing for each sampling date ($P < 0.05$).

Finally, the PCR analysis confirmed the presence of *C. perfringens* in two post mixing samples collected at feed mill A and D. Both samples had the *cpa* gene and did not have the *netB* gene (data not shown).

Discussion:

Microbiological evaluation of animal feeds has become an important element to ensure the quality and safety of the feed that is given to farm animals (Wojdat et al., 2005). The presence of contaminating bacterial agents like *Salmonella*, *E. coli* and *Clostridium perfringens* in animal feeds has been well described by different authors (Dargatz et al., 2005; Maciorowski et al., 2006; Udhayavel et al., 2017). The two major concerns of these pathogens are their potential to produce disease in animals and the possible contamination of the meat with foodborne pathogens that may affect human health (Crump et al., 2002, Sapkota et al., 2007).

Contamination with *Salmonella* serovars in feed mills has been reported not only in feed ingredients and finished feeds but also in dust samples collected at feed mills (Jones et al., 1991). In the samples collected in our study none of the ingredient samples were contaminated with *Salmonella* serovars. These findings agree with Hacking et al. (1978) who did not recover *Salmonella* from ground corn or soybean meal and with Kidd et al. (2002) who did not recover any *Salmonella* serovars from corn gluten and soybean meal samples. Other researchers have been able to recover *Salmonella* serovars from different feed ingredient such as fish meal, meat and bone meal and poultry meal in approximately 36% of the samples analyzed (Jones et al., 1991). Jones and Richardson (2004) were able to detect *Salmonella* positive samples in 5.26% of corn samples and in 10% of the soybean meal samples assayed. The presence for *Salmonella* serovars

in finished feed has been well identified. It has been reported to be recovered from 16-35% of mash feed samples and from 4.3-6.67% of pelleted samples (Hacking et al. 1978; Jones et al., 1991; Jones and Richardson, 2004). A possible reason of why *Salmonella* was not recovered from the samples collected in this study is because a pre-enrichment step in the isolation of the bacterium was not used. If any vegetative bacterial cells of *Salmonella* were present in the ingredient/feed samples, they would have been present in a damaged physiological state due to the heat stress that grains (ingredients) undergo at drying and the high temperatures that feeds undergo prior to pelleting, which impairs the cell's ability to multiply on selective media (Ordal et al., 1976). The pre-enrichment step would allow the physiologically damaged cells to be repaired in a non-selective medium if they were present in the ingredient or feed samples, making them viable for growth on selective media (Juven et al., 1984). The efficacy of this pre-enrichment step in the isolation of *Salmonella* in feeds has been well tested and studies done to recover *Salmonella* from dry feeds showed a higher recovery of the bacterium using a pre-enrichment step with M-9 medium or buffered peptone water (Juven et al., 1984). According to D'Aoust et al. (1992) culturing the samples directly on the enrichment mediums (skipping pre-enrichment) can be counterproductive if the samples contain high levels enteric bacteria other than *Salmonella*. However, Fagerberg and Avens (1976) indicated that the enrichment medium TTB favors the growth of *Salmonella* serovars on a viable vegetative stage and inhibits other enteric bacteria that could be present on the sample. In our study, no pre-enrichment step was used to recover *Salmonella*, but taking into account that no *Salmonella* colonies were present after plating the enrichments onto selective media, we can conclude that the ingredients and feed samples assayed were not contaminated, or at least not contaminated with high numbers of viable vegetative cells of *Salmonella* serovars that could be a potential source of contamination to the animals.

Another common bacterium found in feedstuffs is *E. coli* (Kidd et al., 2002). *E. coli* belongs to a family of bacteria that is commonly found in the lower intestine of birds and mammals (*Enterobacteriaceae)*, therefore its shed in feces. When feces are exposed to oxygen, *E. coli* counts increase exponentially—2-4 logs in 72 hours—and when they are shed into the environment, its spread by migration from feces through the soil into water supplies, such as rivers (Russell and Jarvis, 2001). Feedstuffs may become contaminated with *E. coli* if they come in contact with contaminated agricultural effluents, wildlife feces or if they come in contact with any surface that harbors the bacteria during the harvesting, storage or transportation (Wales et al., 2010). Based on the results of this study it seems that *E. coli* can be present in many ingredients used for animal feed manufacturing for multiple animal species. The mean contamination levels observed in the ingredient samples ranged between 1-4 $\log_{10}$ CFU/gram. On the mixed feed, the samples that displayed higher contamination levels of *E. coli* were the mash samples collected at the post mixing stage of the process and their contamination was about 3 $\log_{10}$ CFU/gram (Table 3.2). These data agrees with findings from other researchers that also recovered *E. coli* from feed ingredient samples and mixed feed samples. In the study conducted by Dargatz et al. (2005) more than 1,000 ingredients and feed samples were assayed to detect *E. coli* and they were able to recover it from more than 48% of the samples assayed. In another study, Lynn et al. (1998) was able to recover *E. coli* from over 30% of the analyzed ingredients and feed samples.

All the samples collected in our study during the processing of the feed were collected at mash stage and then in pellet form. Some of the changes observed between the ECC of the mash feed and the pelleted feed was a significant $\log_{10}$ CFU/g reduction right after the feed went through the pelleting process (with the exception of the samples collected on feed mill B during Apr-Oct that were not significantly lower on the ECC at the post pelleting stage) (Table 3.4). The heat

treatment during conditioning and pelleting reduces microbial pathogens present in the mash feed, but it does not lead to commercial sterility of the feed (U.S. Department of Health and Human Services, 2018). The temperatures required to reduce pathogens range from 70 to 100°C, which includes the temperatures reached during pelleting in the five feed mills sampled, and its intended to kill those microorganisms present in vegetative form, like *E. coli* (U.S. Department of Health and Human Services, 2018). In the data collected, the microbiological reduction on ECC in terms of logs of kill (Table 3.4) was achieved, indicating efficacy of the pelleting process in most of the feed mills sampled (90%). Although heat treatments is one of the most effective methods to control bacterial pathogens, the resulting pelleted feeds are susceptible to be re-contaminated in further steps of the feed processing or transportation to the farms (Maciorowski et al., 2006). In the cases of feed mills, A and B, we can observe that *E. coli* was recovered from samples collected after the heat treatment process (Table 3.4), indicating a possible inefficient pelleting process—referring to the inability to kill pathogens as described by Behnke (1994)—or a possible recontamination in the feed production line. The presence of these bacteria after the heat treatment cannot be directly attributed to a re-contamination of the feed, because no measurements were taken to determine if the actual pelleting process was being successful in killing the bacteria present on the mash feed. Additionally, besides the temperatures that feeds are exposed during pelleting, other factors such as feed formulation, water activity, fat content, pH, salt and protein content may interfere with an effective kill of pathogens (U.S. Department of Health and Human Services, 2018). Therefore, variations in diet formulation may have had an impact over the recovery of *E. coli* after the heat treatment. However, no feed formulations for the samples collected were obtained. For feed mill B (Figure 3.2), it's important to mention that the processing conditions were not adequate for the feed. For example, on both visits it was observed that the feed production process was done with

some damaged equipment which produced feed leaks across the processing line making the environment more likely for contamination, as feed was constantly leaking from the equipment and falling directly to the ground. To reduce the amount of feed waste, all the mixed feeds that fell into the ground were later collected and re-processed in the same production line. Taking into consideration the findings mentioned before from Jones et al. (1991) that *Enterobacteriaceae* can be found on dust collected within the feed mill facilities, it is reasonable to think that the exposure of the mixed feed to the ground may have been a determining factor to recover *E. coli* in samples that were already pelleted.

When analyzing the effect of sampling periods (Tables 3.3 and 3.4) versus their ECC, it can be observed that the date of sample collection did not have a consistent or significant effect over the contamination of the feed ingredients or mixed feeds. The exception to this statement were soybean meal from feed mill C and the post pelleting samples from feed mill B that were in fact different when compared between the sampling periods ($P < 0.05$). The highest ECC for both samples were in the warmest period sampled (Apr-Oct). These results differ from the findings of Jones and Richardson (2004) who found higher *Enterobacteriaceae* counts in the samples collected during colder months (higher in April compared to August). Israelsen and Hansen (1997) suggested that contamination in feeds after pelleting is more likely to happen during cooler months due to the liberation of moisture vapor, which condenses on the cold surfaces of equipment during cooling and transportation. Moisture can trap dust in equipment surfaces creating a suitable environment for pathogen growth, which could contaminate the feed. However, the data collected in our study at post cooling during the cooler months (Nov-Mar) does not support Israelsen and Hansen (1997) suggestion.

Another class of bacteria that are of major concern to the feed industry are the anaerobic bacteria belonging to the genus *Clostridium.* Due to the potential to produce illness, the species of major concern in feed are *C. perfringens* and *C. botulinum*, both are toxin producers which can cause necrotic enteritis and botulism, respectively (Maciorowski et al., 2007). Our results show that contamination with Clostridial spp. is very likely in most of the feedstuffs and mixed feeds produced for different animal species (Tables 3.3 and 3.4). The data collected shows that Clostridial spp. were recovered from 60.6% (80/132) of the feedstuffs sampled and from 84.4% (135/160) of the mixed feeds sampled. *C. perfringens* was confirmed in 9.5% (2/21) of the isolates (see chapter 4 this thesis) that were further assayed in this study and these findings agree with other researchers that also recovered *C. perfringens* from feedstuffs and mixed feed samples, like Casagrande et al. (2013), Wojdat et al. (2005) and Udhayavel et al. (2017) who were able to recover *C. perfringens* from 60, 38, and 33.89% of the samples assayed, respectively.

In general, no effect of sampling period over Clostridial contamination was observed in this study, this means that the contamination of almost all of the feed ingredients and mixed feeds was the same over both sampling periods. It's interesting to note that the feed mills that showed CSC difference in ingredients (Table 3.3) between the two sampling periods (feed mill C-soybean meal and feed mill E-corn meal) did not have any statistical difference on the mixed feed samples collected during those periods. This holds true for those feed mills that had significant difference between sampling periods in the mixed feed samples (Table 3.4) (feed mill A-post mixing and feed mill D-post mixing, post pelleting and post cooling) and did not have any difference over the ingredient samples (Table 3.3). A possible explanation to these inconsistencies is the variation that could have existed between the CSC from the ingredient samples collected (at ingredient receiving, directly from the trucks or trains delivering the ingredients) and the actual ingredients being used

to make the feed that was sampled at the time of the visit (made with ingredients that had been stored in bins for some time prior to use). Additional research is necessary to verify the effect of ingredient storage on the survivability and growth of Clostridial spp.

When comparing the CSC from the four sampling points in the manufacturing process (post mixing, post pelleting, post cooling and loadout) by feed mill (Table 3.4), it was observed that none of the samples were statistically different in the CSC from one stage of processing to the next. This suggests that feed contamination with Clostridial spp. was similar at the beginning and end of the feed manufacturing ($P > 0.05$). This was true for the samples collected on both sampling periods. No effect of the pelleting process was observed because bacteria from the genus *Clostridium* are spore formers and resistant to the pelleting temperatures typically used by the feed industry (Jordan, 2001). According to the U.S. Department of Health and Human Services (2018) temperatures higher than 100°C would be required to kill all form of microorganism, including spores. However, these temperatures cannot be achieved by the feed industry because the matrix and digestibility of nutrients in feed would be damaged and would have deleterious effects over the animals consuming it.

Conclusion:

Animal feeds can be contaminated and serve as a source of transmission of *E. coli* or *Clostridium perfringens* to farm animals. The contamination of animal feeds may lead to disease or infection of the animals which could possibly lead to contamination of the meat and parts in the processing facility with *E. coli* or *Clostridium perfringens*. Thus, mitigation strategies to reduce the contamination of animal feeds should be further evaluated.

References:

Bailey, M.A., Macklin, K.S. and Krehling, J.T. 2013. Use of a Multiplex PCR for the Detection of Toxin-Encoding Genes *netB* and *tpeL* in Strains of *Clostridium perfringens*. International Scholarly Research Notices, 2013.

Behnke, K. C. 1994. Factors affecting pellet quality. Maryland Nutrition Conference. Dept of Poultry Science and animal Science, College of Agricultural, University of Maryland, College Park.

Casagrande, M., Cardozo, M., Beraldo-Massoli, M., Boarini, L., Longo, F., Paulilo, A., and Schocken-Iturrino, R. 2013. *Clostridium perfringens* in ingredients of poultry feed and control of contamination by chemical treatments. Journal of Applied Poultry Research, 22(4), 771-777.

Centers for Disease Control and Prevention (CDC), 2018. *Clostridium perfringens*. Accessed March 2020. Available at https://www.cdc.gov/foodsafety/diseases/clostridium-perfringens.html

Crump, J. A., Griffin, P. M., and Angulo, F. J. 2002. Bacterial contamination of animal feed and its relationship to human foodborne illness. Clinical Infectious Diseases, 35(7), 859-865

D'Aoust, J.Y., Sewell, A.M. and Warburton, D.W. 1992. A comparison of standard cultural methods for the detection of foodborne *Salmonella*. International Journal of Food Microbiology, 16(1), 41-50.

Dargatz, D. A., Strohmeyer, R. A., Morley, P. S., Hyatt, D. R., and Salman, M. D. 2005. Characterization of *Escherichia coli* and *Salmonella enterica* from cattle feed ingredients. Foodbourne Pathogens & Disease, 2(4), 341-347.

Davis, M. A., Hancock, D. D., Rice, D. H., Call, D. R., DiGiacomo, R., Samadpour, M., and Besser, T. E. 2003. Feedstuffs as a vehicle of cattle exposure to *Escherichia coli* O157: H7 and Salmonella enterica. Veterinary Microbiology, 95(3), 199-210.

Economic Research Service (ERS), U.S. Department of Agriculture (USDA), 2014. Cost Estimates of Foodborne Illnesses. Accessed January 2020. Available at https://www.ers.usda.gov/data-products/cost-estimates-of-foodborne-illnesses/cost-estimates-of-foodborne-illnesses/#Pathogen

Fagerberg, D.J. and Avens, J.S., 1976. Enrichment and plating methodology for *Salmonella* detection in food. A review. Journal of Milk and Food Technology, *39*(9), 628-646.

Hacking, W. C., Mitchell, W. R., and Carlson, H. C. 1978. *Salmonella* investigation in an Ontario feed mill. Canadian Journal of Comparative Medicine, 42(4), 400.

Israelsen, M., and Hansen, I. D. 1997. Elimination of *Salmonella* in dry feeds. Iowa State University Digital Repository. Accessed March 2020. Available at https://lib.dr.iastate.edu/cgi/viewcontent.cgi?article=1221&context=safepork

Jones, F.T. and Richardson, K.E., 2004. *Salmonella* in commercially manufactured feeds. Poultry science, 83(3), 384-391.

Jones, F. T., Axtell, R. C., Rives, D. V., Scheideler, S. E., Tarver Jr, F. R., Walker, R. L., and Wineland, M. J. 1991. A survey of *Salmonella* contamination in modern broiler production. Journal of Food Protection, 54(7), 502-507.

Jordan, F. 2001. Clostridia. Pages 154-162 in Poultry Diseases. Jordan, F., Pattison, M., Alexander, D., and Faragher, T., 5th edition. WB Saunders Publishers.

Juven, B. J., Cox, N. A., Bailey, J. S., Thomson, J. E., Charles, O. W., and Shutze, J. V. 1984. Recovery of *Salmonella* from artificially contaminated poultry feeds in non-selective and selective broth media. Journal of Food Protection, 47(4), 299-302.

Keyburn, A. L., Yan, X. X., Bannam, T. L., van Immerseel, F., Rood, J. I., and Moore, R. J. 2010. Association between avian necrotic enteritis and *Clostridium perfringens* strains expressing NetB toxin. Veterinary Research, 41(2), 1-8.

Kidd, R. S., Rossignol, A. M., and Gamroth, M. J. 2002. *Salmonella* and other Enterobacteriaceae in dairy-cow feed ingredients: antimicrobial resistance in western Oregon. Journal of Environmental Health, 64(9), pp. 9.

Lynn, T. V., Hancock, D. D., Besser, T. E., Harrison, J. H., Rice, D. H., Stewart, N. T., and Rowan, L. L. 1998. The occurrence and replication of *Escherichia coli* in cattle feeds. Journal of Dairy Science, 81(4), 1102-1108.

Maciorowski, K. G., Herrera, P., Jones, F. T., Pillai, S. D., and Ricke, S. C. 2007. Effects on poultry and livestock of feed contamination with bacteria and fungi. Animal Feed Science and Technology, 133(1-2), 109-136.

Maciorowski, K. G., Herrera, P., Kundinger, M. M., and Ricke, S. C. 2006. Animal feed production and contamination by foodborne *Salmonella*. Journal für Verbraucherschutz und Lebensmittelsicherheit, 1(3), 197-209.

Ordal, Z. J., Iandola J. J., Ray B., and Sinskey A. G. 1976. Detection and enumeration of injured microorganisms, pp. 163-169. In M. L. Speck (ed.) Compendium of methods for the microbiological examination of foods. American Public Health Association, Washington, DC.

Russell, J.B. and Jarvis, G.N. 2001. Practical mechanisms for interrupting the oral-fecal lifecycle of *Escherichia coli*. Journal of Molecular Microbiology and Biotechnology, 3(2), 265-272.

Sapkota, A. R., Lefferts, L. Y., McKenzie, S., and Walker, P. 2007. What do we feed to food-production animals? A review of animal feed ingredients and their potential impacts on human health. Environmental Health Perspectives, 115(5), 663-670.

Tessari, E. N. C., Cardoso, A. L. S. P., Kanashiro, A. M. I., Stoppa, G. F. Z., Luciano, R. L., and de Castro, A. G. M. 2014. Analysis of the presence of *Clostridium perfringens* in feed and raw material used in poultry production. Food and Nutrition Sciences, 2014.

Tille, P. 2015. *Bailey & Scott's diagnostic microbiology-e-book*. Elsevier Health Sciences.

Udhayavel, S., Ramasamy, G. T., Gowthaman, V., Malmarugan, S., and Senthilvel, K. 2017. Occurrence of *Clostridium perfringens* contamination in poultry feed ingredients: Isolation, identification and its antibiotic sensitivity pattern. Animal Nutrition, 3(3), 309-312.

U.S. Department of Health and Human Services. 2018. Hazard Analysis and Risk-Based Preventive Controls for Food for Animals, Guidance for the Industry. Food and Drug Administration. Center for Veterinary Medicine. Accessed March 2020. Available at https://www.regulations.gov/document?D=FDA-2018-D-0388-0002

van Immerseel, F., Rood, J. I., Moore, R. J., and Titball, R. W. 2009. Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens. Trends in Microbiology, 17(1), 32-36.

Wade, B., and Keyburn, A. 2015. The true cost of necrotic enteritis. Poultry World. Accessed January 2020. Available at https://www.poultryworld.net/Meat/Articles/2015/10/The-true-cost-of-necrotic-enteritis-2699819W/

Wales, A. D., Allen, V. M., and Davies, R. H. 2010. Chemical treatment of animal feed and water for the control of *Salmonella*. Foodborne Pathogens and Disease, 7(1), 3-15.

Walker, J. H. C. 1959. *Salmonella* Organisms in Animal Feeding Stuffs and Fertilizers. Monthly Bull. Ministry of Health & Pub. Health Lab. Service (directed by Med. Res. Council), 18, 26-35.

Wojdat, E., Kwiatek, K., and Kozak, M. 2005. Microbiological quality of animal feedingstuffs in Poland. Bulletin-Veterinary Institute in Pulawy, 49(3), 315.

Wojdat, E., Kwiatek, K., and Kozak, M. 2006. Occurrence and characterization of some *Clostridium* species isolated from animal feedingstuffs. Bulletin-Veterinary Institute in Pulawy, 50(1), 63.

Table 3.1 Characteristics of the feed mills sampled.

Feed Mill	Type of Feed Mill	Prod. Rate (tons/mo.)	Conditioning Temp. (°C)	Types of Feed Sampled (Species)
A	Research	V	73.8 – 76.7	Starter/ Finisher (Broilers)
B	Multi-species	1,500	82.2 – 90.6	Extended Pellet (Cattle)/ Grower (Quail)
C	Integrator	24,000	86.7 – 87.8	Starter/ Withdrawal (Broilers)
D	Integrator	14,000	82.2 – 85.0	Grower 2x (Broilers)
E	Integrator	N/A	85.0 – 87.8	Breeder (Layers)/ Grower (Broilers)

V = Variable production rate
N/A = Not available data

Table 3.2 Mean log_{10}/gram of the *Clostridium* spp. counts (CSC) and *E. coli* counts (ECC) obtained from ingredients and feed samples collected.

Sample	n	CSC	ECC
		Ingredients	
Corn Meal	40	1.94[bc]	2.85[ab]
Soybean Meal	40	0.42[d]	0.49[c]
DDGs	20	0.20[d]	0.92[c]
Poultry by-product meal	16	2.02[bc]	1.34[bc]
Corn Gluten Meal	8	2.61[ab]	2.04[bc]
Peanut Meal	4	3.91[a]	4.15[a]
Wheat	4	1.00[cd]	1.83[bc]
		Process Samples	
Post-Mixer	40	1.95	3.03[a]
Post-Pelleting	40	1.90	0.23[b]
Post-Cooling	40	1.87	0.08[b]
Loadout	40	1.98	0.10[b]

[a-d] Means within a column with different letters are significantly different ($P<0.05$).

Table 3.3 Effect of sampling periods on the mean log_{10}/gram of the *Clostridium* spp. counts (CSC) and *E. coli* counts (ECC) obtained from the ingredient samples collected.

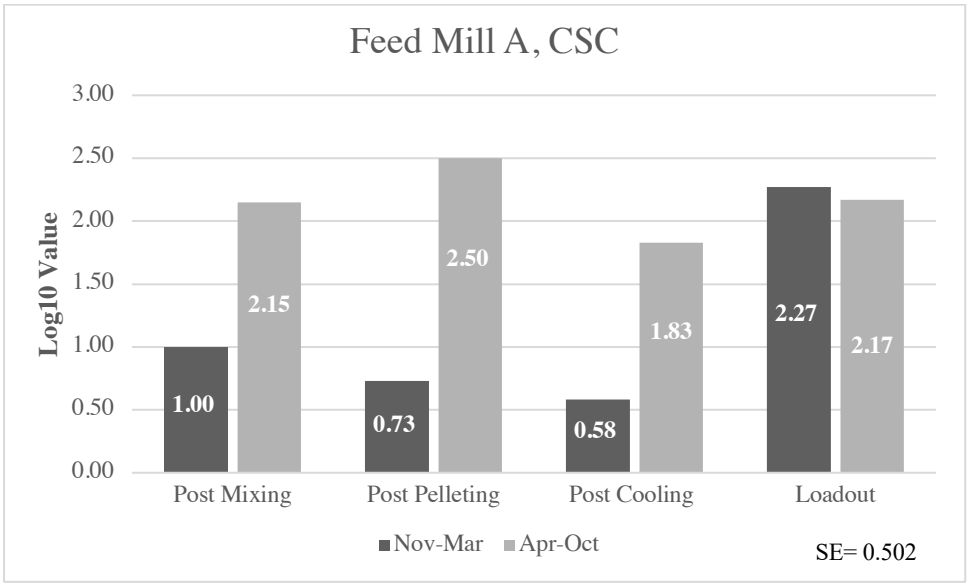
Feed Ingredient/ Feed Mill	n	A	B	C	D	E	A	B	C	D	E
		CSC					ECC				
		Samples Collected Nov-Mar									
Corn Meal	20	0.25^{c}	2.08^{ab}	3.08^{a}	1.26^{bc}	$2.22^{ab(z)}$	2.73	3.12	1.75	3.05	3.49
Soybean Meal	20	0	0	$0^{(z)}$	0	0.76	1.00^{a}	0^{b}	$0.50^{ab(z)}$	0^{b}	0.25^{b}
DDGs	8	0	-	-	-	0.25	$3.61^{(y)}$	-	-	-	0
Poultry by-product meal	8	-	-	-	3.18	1.50	-	-	-	0.75	1.00
Corn Gluten Meal	4	-	-	2.35	-	-	-	-	0.50	-	-
Wheat	4	1.00	-	-	-	-	1.83	-	-	-	-
		Samples Collected Apr-Oct									
Corn Meal	20	1.37^{b}	2.63^{ab}	2.00^{ab}	1.08^{b}	$3.44^{a(y)}$	2.50	2.69	4.08	3.37	1.79
Soybean Meal	20	0^{b}	0.75^{b}	$2.48^{a(y)}$	0^{b}	0.25^{b}	0^{b}	1.00^{ab}	$2.18^{a(y)}$	0^{b}	0^{b}
DDGs	12	0^{b}	0^{b}	-	-	0.75^{a}	$0^{(z)}$	1.00	-	-	0
Poultry by-product meal	8	-	-	-	2.90	0.50	-	-	-	2.12	1.50
Corn Gluten Meal	4	-	-	2.88	-	-	-	-	3.57	-	-
Peanut Meal	4	-	3.91	-	-	-	-	4.14	-	-	-

[a-c] Means within a row by type of bacterium count with different letters are significantly different ($P<0.05$).
[(y,z)] Means within a column with different letters are significantly different ($P<0.05$).
- Indicates that no samples were available for collection, therefore not collected for the study.

Table 3.4 Effect of sampling periods on the mean log_{10}/gram of the *Clostridium* spp. counts (CSC) and *E. coli* counts (ECC) obtained from the stages of processing samples collected.

Feed Sample/ Feed Mill	n	A	B	C	D	E	A	B	C	D	E
		CSC					ECC				
		Samples Collected Nov-Mar									
Post Mixing	20	$1.00^{b(z)}$	2.69^{a}	0.75^{b}	$2.43^{a(y)}$	2.43^{a}	3.09^{ab}	4.84^{a}	1.65^{b}	2.05^{ab}	2.77^{ab}
Post Pelleting	20	0.73	2.39	0.75	$2.12^{(y)}$	2.55	0	$0.25^{(z)}$	0	0	0
Post Cooling	20	0.58^{b}	2.29^{a}	0.50^{b}	$3.22^{a(y)}$	2.56^{a}	0	0	0	0	0
Loadout	20	2.27	2.31	0.58	1.98	2.47	0	0.25	0	0	0
		Samples Collected Apr-Oct									
Post Mixing	20	$2.15^{b(y)}$	3.29^{a}	1.00^{c}	$0.50^{c(z)}$	3.21^{a}	2.50	3.36	4.03	3.42	2.61
Post Pelleting	20	2.50^{b}	3.27^{a}	1.00^{c}	$0.25^{d(z)}$	3.48^{a}	0.50^{b}	$1.58^{a(y)}$	0^{b}	0^{b}	0^{b}
Post Cooling	20	1.83^{b}	3.17^{a}	1.00^{c}	$0.25^{d(z)}$	3.34^{a}	0	0.75	0	0	0
Loadout	20	2.17^{ab}	2.64^{ab}	1.62^{bc}	0.50^{c}	3.21^{a}	0.25	0.50	0	0	0

[a-d] Means within a row by type of bacterium count with different letters are significantly different ($P<0.05$).
[(y,z)] Means within a column with different letters are significantly different ($P<0.05$).



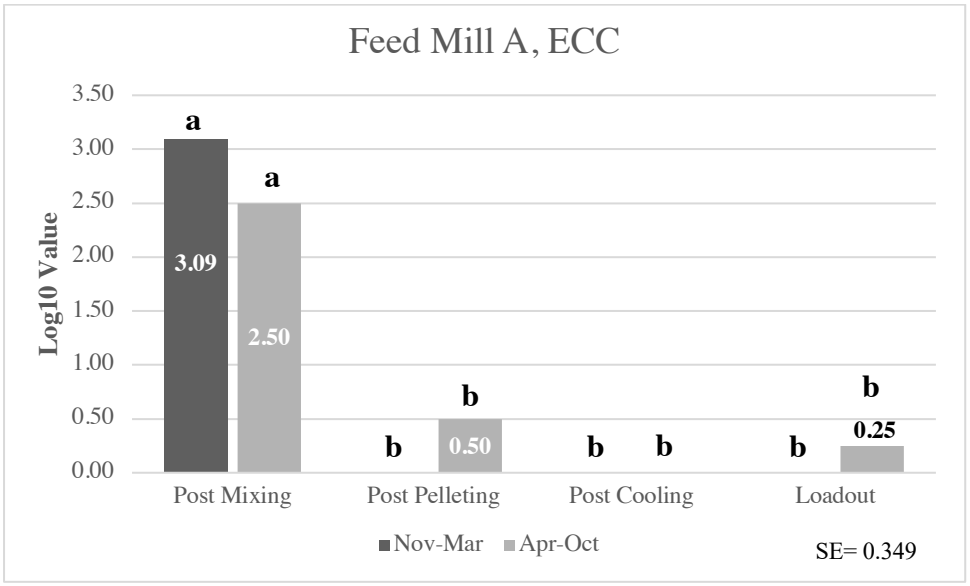
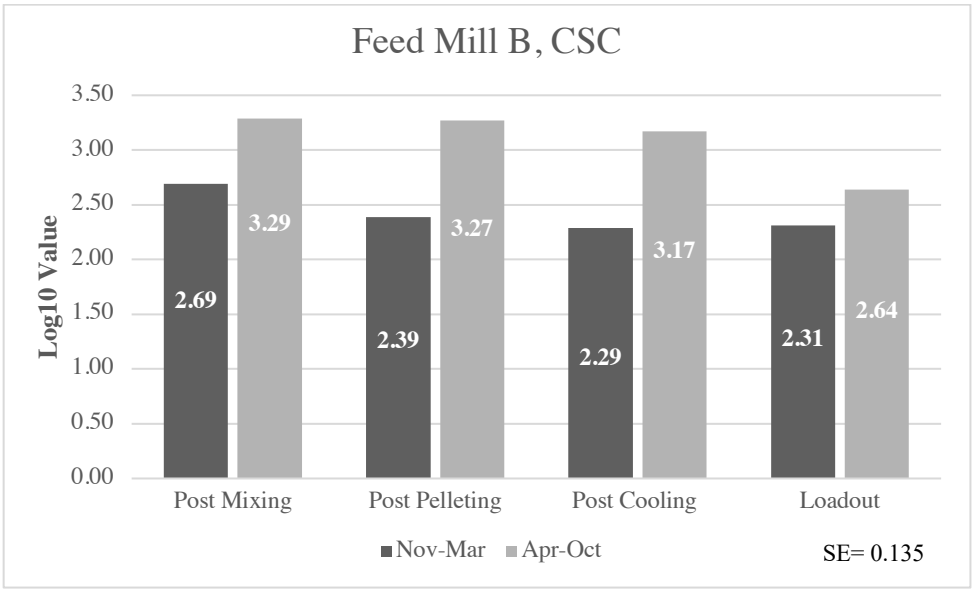


Figure 3.1 *Clostridium* spp. counts (CSC) and *E. coli* counts (ECC) obtained from the stages of processing samples on feed mill A.
[a-b] Indicates significant difference between the stages of processing of the samples analyzed at $P<0.05$ by Tukey's HSD means separation.



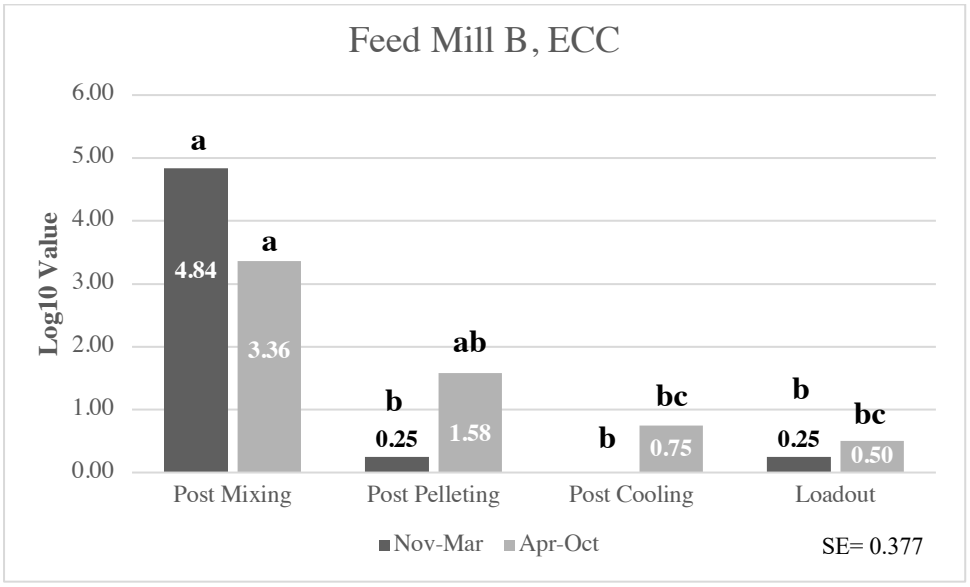
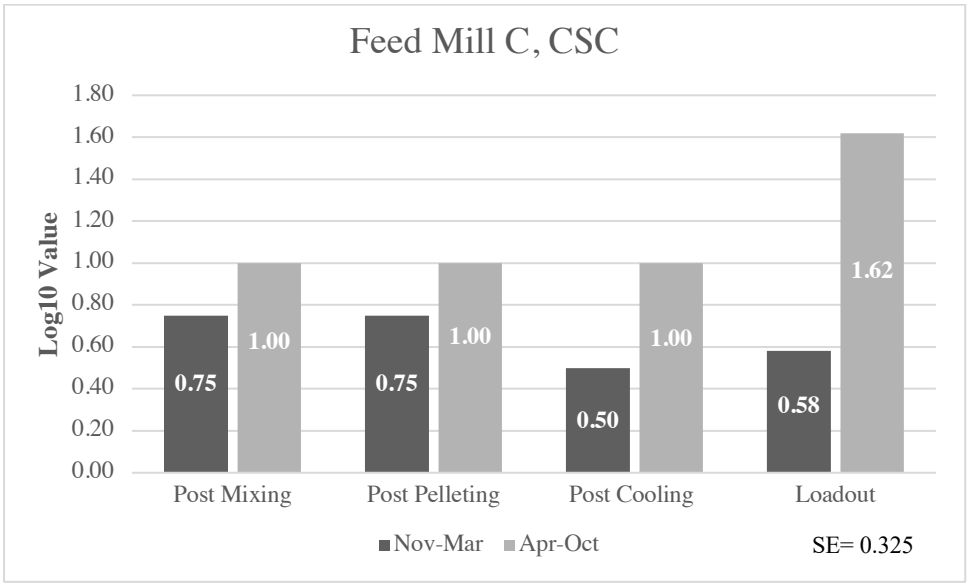


Figure 3.2 *Clostridium* spp. counts (CSC) and *E. coli* counts (ECC) obtained from the stages of processing samples on feed mill B.
[a-c] Indicates significant difference between the stages of processing of the samples analyzed at $P<0.05$ by Tukey's HSD means separation.



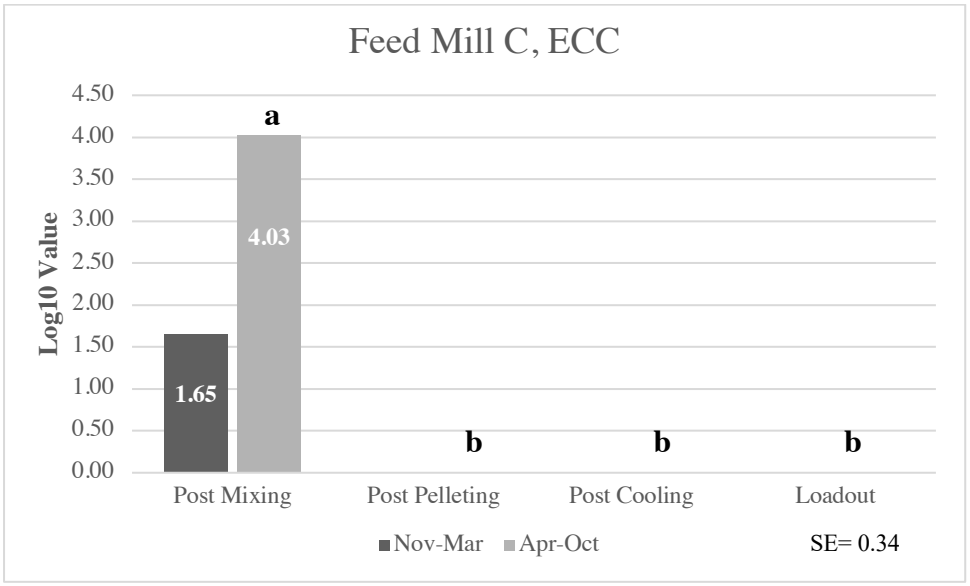
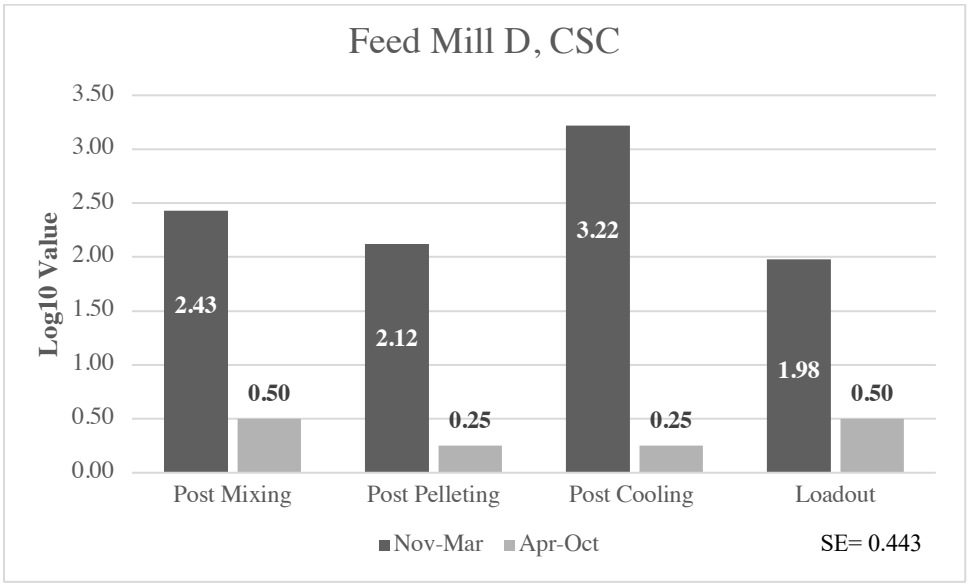


Figure 3.3 *Clostridium* spp. counts (CSC) and *E. coli* counts (ECC) obtained from the stages of processing samples on feed mill C.
[a-b] Indicates significant difference between the stages of processing of the samples analyzed at $P<0.05$ by Tukey's HSD means separation.



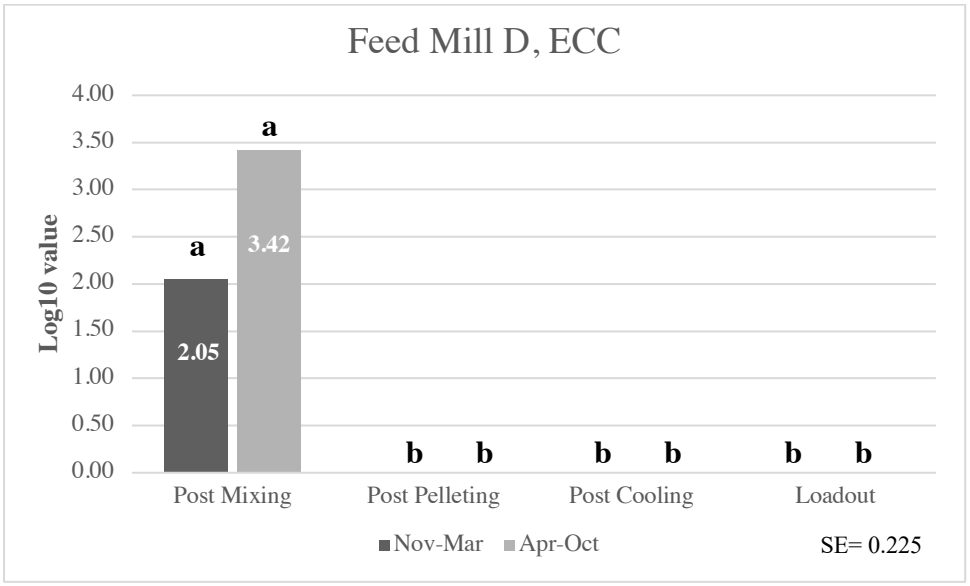


Figure 3.4 *Clostridium* spp. counts (CSC) and *E. coli* counts (ECC) obtained from the stages of processing samples on feed mill D.
[a-b] Indicates significant difference between the stages of processing of the samples analyzed at $P<0.05$ by Tukey's HSD means separation.

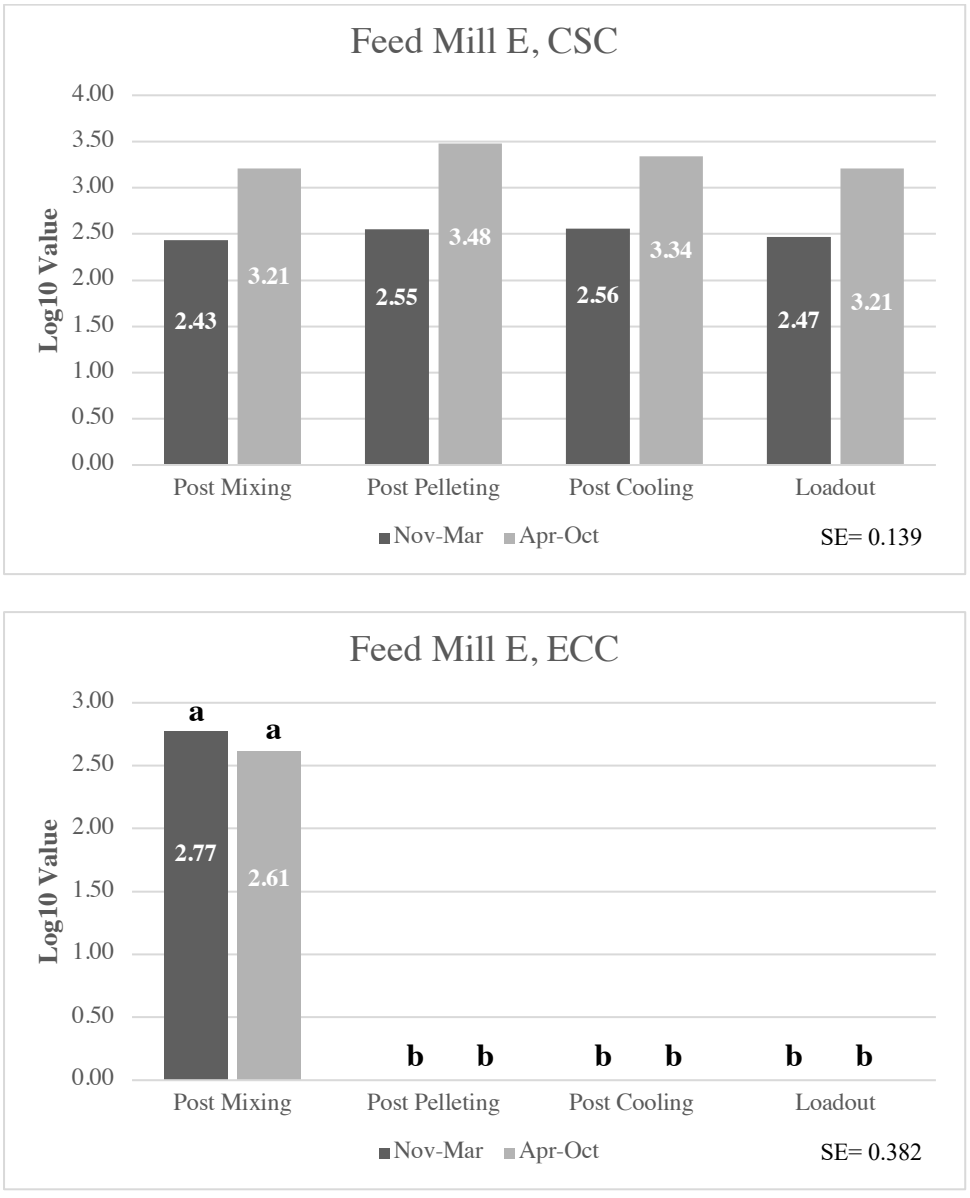


Figure 3.5 *Clostridium* spp. counts (CSC) and *E. coli* counts (ECC) obtained from the stages of processing samples on feed mill E.
[a-b] Indicates significant difference between the stages of processing of the samples analyzed at $P<0.05$ by Tukey's HSD means separation.

Chapter 4.0 Isolation and Identification of an Unknown *Bacillus* spp. from Animal Feeds and a Preliminary Study of its Effect When Used in a Necrotic Enteritis Model

Introduction:

Animal protein products derived from livestock, poultry and fish represent a significant portion of the actual U.S. diet. In 2017, the U.S. non poultry meat production totaled 52 billion pounds and total poultry meat production was 48 billion pounds [North American Meat Institute (NAMI), 2017]. These production levels have been achieved mainly due to nutrition research that has led to improvements in efficiencies and costs of feeds for the producers (Strada et al., 2005). The animal feed industry has played an important role providing all the feed products required to produce these animal proteins. Annually, over 1 billion metric tons of feed are estimated to be produced over the world to supply feed for livestock (bovine and ovine), poultry and swine species [International Feed Industry Federation (IFIF), 2020].

Animal feeds are formulated to provide nutrients that the animals require for their optimal growth. Diets for poultry use corn and soybean meal as major ingredients in their formulation, however the use of other ingredients like animal by-products (i.e. meat and bone meal) and milling by-products (i.e. distillers dried grains with solubles) are often seen in the feed industry because they allow the production of a more economical feed (Sapkota et al, 2007; Casagrande et al., 2013). These ingredients can potentially become contaminated with pathogens from multiple environmental sources during harvest (including dust, soil, water and insects), processing at the feed mill, transportation to the farms or during their storage (Maciorowski et al., 2006). Once contaminated, they serve as vehicles for the pathogens to reach the finished feeds. Since farm animals can acquire pathogens by ingestion, the contamination of mixed feeds can expose the animals to a wide variety of potentially harmful microorganisms, including bacteria which can

produce damage in a clinical or subclinical way at the intestinal level (Crump et al., 2002; Tessari et al., 2014). The feed industry has processes which help to reduce bacterial contamination of feeds, among them is the pelleting process that uses a heat treatment in the conditioning step which is effective killing bacterial pathogens (Behnke, 1994). However, this heat treatment is only effective for those bacteria in their vegetative form, and it doesn't affect those in their spore form. Spore formation is a survival strategy that some bacteria can do in response to environmental conditions (i.e. lack of essential nutrients), which allows the bacterial cell to have a "temporary escape" from the current unfavorable environment (Nicholson et al., 2000). Once the conditions improve, these spores have the ability to germinate and resume the vegetative growth of the bacteria (Nicholson et al., 2000).

There is substantial evidence that spore forming bacteria like Clostridial spp. can be present in animal feeds, thus becoming a source of potential contamination to farm animals. Our previous study allowed us to confirm *Clostridium perfringens* from feed samples collected over five different feed mills. Additionally, we were able to identify two unknown groups of bacteria that had similar characteristics to those from the *Clostridium* genus—rod-shaped, gram positive, grew under anaerobic conditions, formed spores and some isolates were lecithinase positive—. Some of the isolates displayed double-zone beta hemolysis on blood agar which presumed that the isolates were *Clostridium perfringens*, however, when tested at a molecular level by polymerase chain reaction (PCR), the bacterium did not have the alpha-toxin gene characteristic of *Clostridium perfringens*. Therefore, further investigation was warranted to identify the unknown groups of bacteria isolated from the feed samples and determine if they are able to cause disease in birds. Consequently, the objective of this study was to identify and classify these unknown species of

bacteria found in animal feeds and determine their pathogenic potential on birds under a necrotic enteritis model.

Materials and Methods:

Isolates Selection

From the feed samples collected in chapter 3, a single bacterial colony from every sample that showed beta hemolysis on Tryptic Soy Agar II plates containing 5% Sheep Blood (TSA+5% SB; BD BBL™, Becton, Dickinson, and Company, Sparks, MD, USA) was selected to be further analyzed in this study. For those bacterial colonies that displayed alpha hemolysis on TSA+5% SB, a visual evaluation was performed and for those samples collected within the same feed mill and same sampling point that had similar colony morphology, a single colony was selected to be further analyzed. All selected bacterial colonies were cultured in Brain Heart Infusion Broth (BHIB; Criterion™, Hardy Diagnostics, Santa Maria, CA, USA) with 20% glycerol, and frozen in cryovials with sterile beads at -80°C.

Bacterial Staining

From the selected isolates stored in cryovials, one or two beads was selected and placed onto a pre-reduced TSA+5% SB. Then they were incubated in the anaerobic chamber at 40°C for 24 hours. The bacterial cultures from each selected isolate were then classified by their morphologic structure and bacterial cell wall using a Gram Stain Set (Harleco®, EMD Performance Materials, Gibbstown, NJ, USA). A clean glass slide was used to suspend a bacterial colony into a droplet of water. After spreading the bacterial colony evenly into the slide, the smear was allowed to air dry and then it was heat fixed by flaming using a burner. Once that the smear was heat fixed, the slide was allowed to cool down to room temperature and it was flooded with Crystal Violet for 1 minute and rinsed using distilled water. Then the slide was flooded with Gram

Iodine Solution for 1 minute and rinsed using distilled water. The slide was then rinsed with 99% ethanol for 15 seconds and then it was washed thoroughly using distilled water. The slide was then flooded with Safranine Stain for 1 minute and rinsed with distilled water. Finally, the slide was dried using absorbent paper and placed into the microscope for morphological and Gram stain classification. Bacterial cells stained with a purple color were classified as Gram positive and bacterial cells with a pink to red color were classified as Gram negative.

Spore Formation Test

The isolates obtained from feed samples collected from chapter 3 that were selected to be further analyzed, were transferred to separate BHIB tubes (5 mL) and then incubated in the anaerobic chamber (containing 5% CO_2, 5% H_2 90% N_2) for 24 hours at 40°C. After incubation, 1 mL from each culture was transferred into a micro centrifuge tube and then the cultures were placed into boiling water for 15 minutes. From each culture, 100 µL were spread plated in duplicate into pre-reduced TSA + 5% SB plates and incubated anaerobically for 24 hours at 40°C. The isolates that grew colonies after the incubation period were considered positive for spore formation and those isolates that did not grow on the TSA + 5% SB plates were classified as negative.

Lecithinase Test

The isolates obtained from feed samples collected from chapter 3 that were selected to be further analyzed, were streaked into Tryptose Sulfite Cycloserine agar (TSC) plates containing egg yolk emulsion. Then the plates were anaerobically incubated for 24-48 hours at 40°C. After incubation, those isolates that produced a white color turbidity around the bacterial colonies were considered as lecithinase positive and those which did not have the white turbidity around the bacterial colonies were considered as lecithinase negative.

RapID™ ANA II Test

From the selected isolates stored in cryovials, one or two beads was selected and placed onto a pre-reduced TSA+5% SB. Then they were incubated in the anaerobic chamber at 40°C for 24 hours. The bacterial cultures of some of the selected isolates were then tested using a RapID ANA II Test (Thermo Scientific™, Lenexa, KS, USA). For the inoculum preparation, a loop was used to suspend enough bacterial colonies from each isolate into separate tubes of RapID Inoculation Fluid (1 mL) to achieve a visual turbidity equal to a #3 McFarland turbidity standard. The inoculation of the RapID ANA II Panels was performed according to the manufacturer instructions. The inoculated panels were then aerobically incubated at 37°C for 4 hours. The scoring of the RapID ANA II Panels was performed according to manufacturer instructions and the numerical coding obtained from the test was used in the ERIC® data base for the test-isolate identification.

DNA Extraction and 16S rRNA Gene Sequencing

Isolates obtained from the feed samples collected from chapter 3 were stored at -80°C in cryovials with sterile beads containing BHIB + 20% glycerol. From these cryovials, one or two beads was selected and placed onto a pre-reduced TSA+5% SB. Then they were incubated in the anaerobic chamber at 40°C for 24 hours. A single colony displaying either double-zone beta hemolysis or alpha hemolysis was chosen and transferred into 5 mL of BHIB and then incubated in the anaerobic chamber at 40°C for 24 hours. The bacterial DNA from each isolate was then extracted using the Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA) according to the manufacturer instructions. The extracted DNA was then tested for concentration and purity using 1.5 μL of each DNA extraction using a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, Bremen, Germany) according to manufacturer instructions. The standard for purity was a 260nm/280nm ratio between 1.8-2.0. The extracted

DNA was then refrigerated at 4°C until it was shipped to Genewiz® (South Plainfield, NJ, USA) for a 16S ribosomal RNA gene sequencing. The sequence analyses were performed by Genewiz® and the output of their analyses were the best genus and species classification of each isolate.

Broilers and Farm Management

Day-old female broiler chicks were obtained from a commercial hatchery and placed at the Auburn University Poultry Research Farm in Auburn, AL. Upon arrival, the chicks were weighed and randomly distributed throughout four Petersime wire batteries (4 cages, 10 birds per cage) in a temperature-controlled room maintained at 32.2 ± 1°C during the first week and reduced 2-3°C weekly, for four weeks. The birds were fed a two-phase feeding program, consisting of a crumble starter diet (day 0 to 14) and a pelleted grower diet (day 14 to 28). Diets did not contain antibiotics or anticoccidials. Birds were allowed *ad libitum* access to both feed and water throughout the duration of the experiment (28 days total).

Challenge Scheme

At day 18 of age, broilers were given a 1 mL oral gavage of a 20x coccidiosis vaccine (ADVENT®). From day 21–23, a 1 mL oral gavage of *Clostridium perfringens* or *Bacillus proteolyticus* was administered to the appropriate treatments. Intestinal lesion scoring was performed during necropsy, 10 days post-*Eimeria* challenge as described below.

Eimeria Challenge

A 10,000-dose vial (10 mL) of the ADVENT® coccidiosis vaccine (Huvepharma, Peachtree City, GA, USA) was diluted with 490 mL of phosphate buffered saline (PBS) to administer a 20x of the recommended dose per bird. The ADVENT® coccidiosis vaccine contained live oocysts of *Eimeria acervulina*, *Eimeria maxima* and *Eimeria tenella*. From the diluted solution, 1 mL was given to the birds via an oral gavage on day 18.

Clostridium perfringens and *Bacillus proteolyticus* Challenge

Clostridium perfringens (CP) and *Bacillus proteolyticus* (BP) isolates were used for this experiment. Beads of CP and BP isolates held in a -80°C freezer were placed onto a pre-reduced TSA+5% SB plate or onto a pre-reduced Tryptic Soy Agar (TSA; Criterion™, Hardy Diagnostics, Santa Maria, CA, USA) plate containing 5% Rabbit Blood (RB; Hemostat Laboratories, Dixon, CA, USA). After incubation under anaerobic conditions for 24 hours at 40°C, 1-2 colonies displaying beta hemolysis, with typical CP and BP morphologies, were transferred to separate BHIB tubes (100 mL) and then incubated anaerobically for 24 hours at 40°C. After incubation, 1 mL of the culture was diluted with 99 mL of PBS to produce an approximately 10^6 CFU/mL inoculum. The inoculum was orally administered to broilers of the challenged treatments groups on days 21, 22, and 23. Fresh inoculum was prepared for each challenge day. Inoculum levels were verified by serially diluting and spread plating the inoculum on duplicate onto TSA+5% SB plates, incubated anaerobically for 24 hours at 40°C and counting viable colonies showing typical colony morphology.

Data Collection

Body weight (BW) was measured on days 0, 14 and 28. Feed consumption (FC) was measured on days 14 and 28. Any mortalities were removed, weighed and recorded on a daily basis. At day 28, all the remaining birds from each pen were euthanized by CO_2 gas inhalation and then necropsied to score lesions present on the intestinal tract. Enteritis lesions were scored in the duodenum, jejunum, and ileum using a modified necrotic enteritis (NE) scale described by Prescott et al. (1978). Lesions were scored on a scale of 0 to 4, where 0 = no apparent lesions; 1 = thin friable intestines; 2 = focal necrosis, ulceration or both; 3 = patchy necrosis; and 4 = severe

extensive mucosal necrosis. Coccidiosis lesions were scored according to the methods described by Johnson and Reid (1970). A description of these scores are presented in table 4.1.

Data Analysis

All statistical analysis, with the exception of the 16S rRNA gene sequencing, were conducted using IBM® SPSS® software version 26. Data pertaining to the NE intestinal lesion scoring, BW and FCR were analyzed by using a General Linear Model (GLM). Significant differences were reported at $P < 0.05$, and if applicable, means were separated using Tukey's HSD.

Results:

The morphological properties and the biochemical tests performed to identify the selected unknown bacterial species from the feed samples collected over five feed mills are presented on table 4.2. This table shows that all the isolates that were further assayed had the same colony morphology (rods) and that they were positive for Gram's reaction, with the exception of the isolate recovered from the poultry by-product meal sample (feed mill D). The majority of the isolates were spore formers, however, those that were not, had been collected during the manufacturing process at post mixing (feed mill B), post pelleting (feed mill C), post cooling (feed mill D and E) or in the ingredients corn gluten meal (feed mill C) and poultry by-product meal (feed mill D). All the isolates were lecithinase positive, with the exception of the isolates from post mixing (feed mill B) and post pelleting (feed mill E). The isolates displaying beta hemolysis on TSA+5% SB were recovered from samples collected during the manufacturing process at post mixing (feed mills A and D), post pelleting (feed mill B), post cooling and loadout (feed mill A) or in the ingredient corn gluten meal (feed mill C). The isolates displaying alpha hemolysis on TSA+5% SB were recovered from samples collected during the manufacturing process at post mixing (feed mills B and E), post pelleting (feed mills C and E), post cooling (feed mills B, D and

E), loadout (feed mill E) or in the ingredients corn meal (feed mill E) and poultry by product meal (feed mill D). Finally, the Clostridial spp. identified by the RapID ANA II Test on the isolates evaluated were *C. innocuum* (corn gluten meal, feed mill C; post cooling, feed mill D), *C. perfringens* (post mixing, feed mills A and D) and *C. subterminale* (post pelleting, feed mills B and C; post cooling, feed mill E). Both *C. perfringens* isolates were *cpa* positive and *netB* negative (data not shown).

Table 4.3 illustrates the isolates selected for 16S rRNA gene sequencing from the isolates previously described (Table 4.2). The selected isolates had certain similarities with *C. perfringens* (i.e. their ability to grow under anaerobic conditions, their cellular morphology—rod-shaped; their gram stain reaction—gram positive; and in most cases, their ability to produce lecithinase and form spores as shown on table 4.2) and they were recovered from four of the five feed mills previously sampled. No isolates from feed mill C were sequenced because very few of the suspect Clostridial spp. displayed typical colonies on TSC agar and showed hemolysis when streaked onto TSA+5% SB. The 16S rRNA gene sequencing confirmed the presence of three species of bacteria, *Bacillus proteolyticus* (post cooling and loadout, feed mill A), *Clostridium argentinense* (post pelleting, feed mills B and E; post cooling, feed mills B, D and E; loadout, feed mill E) and *Clostridium perfringens* (post mixing, feed mills A and D).

The growth performance of the broilers challenged with *Eimeria* spp. and *C. perfringens* or *B. proteolyticus* is shown in table 4.4. Body weight (BW) gain did not show any statistical difference between the treatments evaluated ($P > 0.05$), the treatment with the highest BW at the end of the trial (day 28) was the one challenged with *Eimeria* spp. and *C. perfringens* (14.10 Kg/10 birds), followed by the unchallenged control treatment (12.90), cocci challenged control treatment (12.88) and the treatment challenged with *Eimeria* spp. and *B. proteolitycus* (12.62). The feed

consumption (FC) had the same distribution as the BW; at day 28 the treatment that consumed the most feed were those challenged with *Eimeria* spp. and *C. perfringens* (21.40 Kg/10 birds), followed by unchallenged control (20.28), cocci challenged control (20.18) and the treatment that consumed the least feed was the challenged with *Eimeria* spp. and *B. proteolyticus* treatment (18.60). For feed conversion ratio (FCR) no statistical difference were observed ($P > 0.05$), the treatment that had the lowest FCR was the one challenged with *Eimeria* spp. and *B. proteolyticus* (1.4739 Kg of feed/Kg of BW gain), followed by the treatment challenged with *Eimeria* spp. and *C. perfringens* (1.5177), cocci challenged control (1.5668) and the group with the highest FCR was the unchallenged control (1.5721). No mortality occurred during the duration of the trial.

The results for necrotic enteritis (NE) are shown on table 4.5. The highest NE score observed in the trial was a 1, and the treatment that had the highest number of birds with lesions was the one co-infected with *Eimeria* spp. and *C. perfringens* (4). The rest of the treatments (unchallenged control, cocci challenged control, and the one co-infected with *Eimeria* spp. and *B. proteolyticus*) had only 1 bird with a lesion score of 1. No statistical differences were observed ($P > 0.05$) between the treatments evaluated in the NE scores. For the coccidiosis evaluation, only a small portion of the birds showed minor intestinal changes attributable to coccidiosis, therefore that data is not included in this study.

Discussion:

The previous study allowed us to identify the presence of an unknown suspect Clostridial spp. in feed ingredients and finished feed collected from five different feed mills. The present study showed through 16S rRNA gene sequencing analysis that the suspect Clostridial spp. in the samples collected was *Clostridium argentinense*. Additionally, this assay also confirmed the

presence of *Bacillus proteolyticus* on samples collected from feed mill A, the research feed mill from Auburn University.

As observed in table 4.3, all the bacterial species found through the 16S rRNA gene sequencing analysis are species that have the ability to produce spores, therefore, they have the ability to survive the temperatures and steam pressure of the conditioning process prior to pelleting. From the ten isolates sent for sequencing, eight were recovered from samples collected after the pelleting process and the remaining two isolates were recovered from samples collected at the post mixing stages of feed mills A and D. For these last two isolates, it was confirmed that the bacterium present in the feed samples was *C. perfringens*. This sequencing results, confirm our previous findings which identified those same isolates as *C. perfringens* by the RapID ANA II Test (Table 4.2) and PCR (not shown). However, the results from this study were not able to prove the presence of *C. perfringens* in subsequent stages of processing at feed mills A or D. These findings could be explained by the fact that only four black colonies from the selective media (TSC) were tested for their ability to produce a double-zone beta hemolysis on TSA+5% SB. Those samples collected from subsequent stages of processing at feed mills A and D either did not show beta hemolysis on TSA+5% SB (48 isolates) or did not have the *cpa* and *netB* genes (20 isolates). The black colony selection factor could have played a role on why no *C. perfringens* was recovered from samples after pelleting in those feed mills contaminated at the post mixing stage of processing.

The second species of bacteria identified through the gene sequencing analysis was *Bacillus proteolyticus.* This bacterium was found on the samples collected at feed mill A (Auburn University's research feed mill) at the stages of processing of post cooling and loadout. *B. proteolyticus* was described by Liu et al. (2017) as a spore-forming bacterium that is able to grow under anaerobic conditions (facultative anaerobe), and as other members of the "*Bacillus cereus*

group" it has the potential to produce double-zone beta hemolysis on TSA+5% SB, characteristics that were in fact observed in this study. *B. proteolyticus* has been recovered from diverse marine environments, such as ocean sediment and fish processing waste from fresh and marine water (Bhaskar et al., 2007). Therefore, the presence of this bacterium on ingredients, such as fish meal, is likely. The diets sampled for the present study from feed mill A did not contain fish meal. However, research for other animal species that included fish meal as part of the diet formulation has been performed at feed mill A, giving a reasonable explanation for the origin of the contamination with *B. proteolyticus* found in this study. To our knowledge, there is no previous studies indicating that *B. proteolyticus* could be pathogenic to animals or humans, however since it has the capability of producing an alkaline protease it is possible that it may induce dermatitis or respiratory ailments (Boyer and Byng, 1996; Logan, 1988).

The third bacterium identified by 16S gene sequencing analysis was *Clostridium argentinense*. It was observed (Table 4.3) that *C. argentinense* was recovered from samples collected across the manufacturing processes (except post mixing stage) of the different feed mills, which indicates that the bacterium has a resistance to high temperatures and can survive the pelleting process. These findings can be attributed to the ability of this bacterium to produce spores (Suen et al., 1988). The contamination of multiple feed mills (B, D and E) indicates that the source of the bacterium is more likely to be a contaminated ingredient than inadequate feed mill sanitation. The possible contamination of ingredients with *C. argentinense* may occur during the harvest of the grains, as it is known that this bacterium is ubiquitous to soil and it may contaminate the grains through wind, rain or mechanical harvesting (Gimenez and Ciccarelli, 1970). In regard to their ability to produce hemolysis, it was observed that the *C. argentinense* isolates found in this study did not produce a strong hemolysis on sheep blood (when it did, it was an alpha hemolysis).

However, on TSA+5% RB the isolates produced double zone beta hemolysis, results that agree to those found by Gimenez and Ciccarelli (1970). It is interesting to point out that from the six isolates that were confirmed as *C. argentinense* by 16S sequencing (Table 4.3), two (post cooling stages, feed mills D and E) did not form spores (Table 4.2). These findings agree with those made by Gimenez and Ciccarelli (1970) in which it was indicated that spores were rarely formed by this organism and with Suen et al. (1988), which indicated that the bacterium may or may not have the ability to produce spores. In this study four of the six bacteria identified as *C. argentinense* produced spores.

C. argentinense has the potential to produce a neuroparalytic toxin that can cause botulism (toxin group G) (Suen et al., 1988). Neurotoxins are responsible of causing severe acute neuroparalytic disease, which produce paralysis and pulmonary arrest in humans, birds and other animals (Ghanem et al., 1991). For *C. argentinense*, it has been observed since the first characterization of the bacterium that not all the isolates are capable of producing this neurotoxin (Gimenez and Ciccarelli, 1970). A study conducted by Eklund et al. (1988) suggested that the production of the neurotoxin of *C. argentinense* is mediated by the presence of an 81-megadalton plasmid. The ability of producing the neurotoxin or not determines this bacterium's pathogenicity. Whether they contain the plasmid or not, all other biochemical, metabolic and physiological characteristics are the same (Lewis et al., 1981; Eklund et al., 1988). These findings suggest that the structural gene for toxin production or a regulatory element that influences the toxin synthesis are present on the plasmid (Eklund et al., 1988). Smith (1977) proposed that *C. argentinense* has a phenotypically similar non-toxigenic counterpart, *C. subterminale*. In that case, all those *C. argentinense* isolates that do not produce the neurotoxin can be identified as *C. subterminale* (Eklund et al., 1988). This would explain why in our study the isolates obtained from the post

pelleting stage of process (feed mill B) and post cooling stage of processing (feed mill E) were identified as *C. subterminale* by the RapID ANA II Test but confirmed as *C. argentinense* by the 16S sequencing analysis (Tables 4.2 and 4.3). Further evaluation of the isolates in this study is required to determine if the species identified as *C. argentinense* by 16S sequencing (Table 4.3) have the plasmid that is involved in the neurotoxin production or not. The symptoms observed in the animals infected with the neurotoxin produced by *C. argentinense* are the same symptoms produced by the known toxins of *C. botulinum* types A – F (Ciccarelli et al., 1977). Several animal species have been reported to be susceptible to the neurotoxin produced by *C. argentinense*, and for poultry, the most noticeable clinical signs are muscular weakness in the legs and neck, paralysis (as the illness progress), limberneck and death (Ciccarelli et al., 1977). Up to date, no botulism outbreaks produced by *C. argentinense*'s neurotoxin have been reported in any animal species or in humans, however, the susceptibility of monkeys and chickens when orally given the neurotoxin creates a concern about human susceptibility (Eklund et al., 1988).

There was no effect on growth of birds in any of the treatments (Table 4.4) when comparing the co-infected birds (*Eimeria* spp. and *C. perfringens* or *B. proteolyticus*) to the unchallenged ones. Both of the co-infected groups *Eimeria* spp. and *C. perfringens* or *B. proteolyticus*, had the best BW gain (14.10) and FCR (1.4739), respectively. The findings in combination to the absence of clinical signs (i.e. depression, decreased appetite, diarrhea, inefficient feed utilization, impaired growth rate) suggests that co-infected groups did not develop any acute or subacute infections when given a 20x dose of a coccidiosis vaccine or when orally challenging the birds with *C. perfringens* or *B. proteolyticus*. These findings are further substantiated in that the cocci challenged control had similar results to the unchallenged control, implying that the cocci challenge was insufficient to cause disease.

Based on the NE lesion scores in table 4.5, it can be appreciated that the greatest damage observed within the gastrointestinal tract was thin friable intestines in 7/40 birds. No necrosis (lesion scores 2 or higher) was observed in the trial. There are a few possibilities as to why the challenged birds did not develop any clinical signs. The first factor that could've played a role is the low pathogenicity of the coccidiosis vaccine used, even at 20x of the recommended dose no significant coccidiosis gross lesions were observed in the duodenum, jejunum or ceca at the end of the trial (day 28), which is 10 days post coccidiosis challenge. Considering that under experimental conditions the mucosal damage caused by the co-infection with *Eimeria* spp. is one of the most critical factors involved in NE development, it is not surprising that latter challenge with *C. perfringens* did not result in severe NE lesions (2 > scores). For *B. proteolyticus* there is no evidence that suggests that this bacterium can produce NE in birds and the data found in our study suggest that this bacterium, or at least the strain utilized in this study, does not have the potential to induce enteritis in birds. A second factor that could have played a role, is the *C. perfringens* isolate that was used to inoculate the birds. A study performed by Timbermont et al. (2009) suggested that high numbers of *C. perfringens*—that have been previously confirmed to produce alpha toxin is not sufficient to induce to NE in birds with coccidiosis, but that the ability to produce NE is dependent of the origin of the strain, were those who produce disease are isolates that come from broilers with NE and not from strains isolated from healthy birds. In our case, the strain of *C. perfringens* used to challenge the birds was one isolated from the feed samples collected at the post mixing stage of processing of feed mill D. It can be observed in table 4.5 that 4/10 birds of the treatment challenged with *Eimeria* spp. and *C. perfringens* showed lesion scores of 1, which does not eliminate the chance that this isolate cannot cause NE in predisposed birds. Therefore, further experimental evaluations of the isolates used in this study is required to

determine if the strains of *C. perfringens*, *C. argentinense* or *B. proteolyticus* found in samples collected from feed mills are able to induce to disease in birds that have coccidiosis.

Conclusion:

The unknown presumptive Clostridial spp. found in animal feeds were *Clostridium argentinense* and *Bacillus proteolyticus*. In a co-infected coccidiosis model, *B. proteolyticus* did not produce signs of necrotic lesions in chickens, however, a strain of *C. perfringens* recovered from animal feeds was able to induce mild NE lesions. Further research of the pathogenicity of the *C. perfringens* and *C. argentinense* isolates recovered from animal feeds is necessary to determine their role on disease transmission to farm animals.

References:

Behnke, K. C. 1994. Factors affecting pellet quality. Maryland Nutrition Conference. Dept of Poultry Science and animal Science, College of Agricultural, University of Maryland, College Park.

Bhaskar, N., Sudeepa, E., Rashmi, H., and Selvi, A. 2007. Partial purification and characterization of protease of *Bacillus proteolyticus* CFR3001 isolated from fish processing waste and its antibacterial activities. Bioresource Technology, 98(14), 2758-2764.

Boyer, E.W. and Byng, G.S. 1996. *Bacillus proteolyticus* species which produce an alkaline protease. U.S. Patent 5,518,917.

Casagrande, M., Cardozo, M., Beraldo-Massoli, M., Boarini, L., Longo, F., Paulilo, A., and Schocken-Iturrino, R. 2013. *Clostridium perfringens* in ingredients of poultry feed and control of contamination by chemical treatments. Journal of Applied Poultry Research, 22(4), 771-777.

Ciccarelli, A., Whaley, D., McCroskey, L., Gimenez, D., Dowell, V., and Hatheway, C. 1977. Cultural and physiological characteristics of *Clostridium botulinum* type G and the susceptibility of certain animals to its toxin. Applied and Environmental Microbiology, 34(6), 843-848.

Crump, J. A., Griffin, P. M., and Angulo, F. J. 2002. Bacterial contamination of animal feed and its relationship to human foodborne illness. Clinical Infectious Diseases, 35(7), 859-865.

Eklund, M.W., Poysky, F.T., Mseitif, L.M. and Strom, M.S. 1988. Evidence for plasmid-mediated toxin and bacteriocin production in *Clostridium botulinum* type G. Applied and Environmental Microbiology, 54(6), 1405-1408.

Ghanem, F.M., Ridpath, A.C., Moore, W.E. and Moore, L.V. 1991. Identification of *Clostridium botulinum, Clostridium argentinense*, and related organisms by cellular fatty acid analysis. Journal of Clinical Microbiology, 29(6), 1114-1124.

Gimenez, D., and Ciccarelli, A. S. 1970. Another type of *Clostridium botulinum*. Zentralblatt fur Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, Abt. I (Originale), 215(2), 221-4.

International Feed Industry Federation (IFIF), 2020. The global feed industry. Accessed March 2020. Available at https://ifif.org/global-feed/industry/

Johnson, J. and Reid, W.M. 1970. Anticoccidial drugs: lesion scoring techniques in battery and floor-pen experiments with chickens. Experimental parasitology, 28(1), pp. 30-36.

Lewis, G.E., Kulinski, S.S., Reichard, D.W. and Metzger, J.F. 1981. Detection of *Clostridium botulinum* type G toxin by enzyme-linked immunosorbent assay. Applied and Environmental Microbiology, 42(6), 1018-1022.

Liu, Y., Du, J., Lai, Q., Zeng, R., Ye, D., Xu, J., and Shao, Z. 2017. Proposal of nine novel species of the *Bacillus cereus* group. International Journal of Systematic and Evolutionary Microbiology, 67(8), 2499-2508.

Logan, N.A. 1988. *Bacillus* species of medical and veterinary importance. Journal of Medical Microbiology, 25(3), 157-165.

Maciorowski, K. G., Herrera, P., Kundinger, M. M., and Ricke, S. C. 2006. Animal feed production and contamination by foodborne *Salmonella*. Journal für Verbraucherschutz und Lebensmittelsicherheit, 1(3), 197-209.

Nicholson, W. L., Munakata, N., Horneck, G., Melosh, H. J., and Setlow, P. 2000. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiology and Molecular Biology Reviews, 64(3), 548-572.

North American Meat Institute (NAMI), 2017. The United States meat industry at glance. Accessed March 2020. Available at https://www.meatinstitute.org/index.php?ht=d/sp/i/47465/pid/47465

Prescott, J. F., Sivendra, R., and Barnum, D. A. 1978. The use of bacitracin in the prevention and treatment of experimentally induced necrotic enteritis in the chicken. The Canadian Veterinary Journal, 19(7), 181.

Sapkota, A. R., Lefferts, L. Y., McKenzie, S., and Walker, P. 2007. What do we feed to food-production animals? A review of animal feed ingredients and their potential impacts on human health. Environmental Health Perspectives, 115(5), 663-670.

Smith, L.D. 1977. Botulism. The organism, its toxins, the disease. Charles C. Thomas, 301-327 East Lawrence Avenue Springfield, Illinois 62717, USA.

Strada, E., Abreu, R.D., Oliveira, G., Costa, M., Carvalho, G., Franca, A.S., Clarton, L. and Azevedo, J., 2005. Uso de enzimas na alimentação de frangos de corte. Revista Brasileira de Zootecnia, 34(6), 2369-2375.

Suen, J. C., Hatheway, C. L., Steigerwalt, A. G., and Brenner, D. J. 1988. *Clostridium argentinense* sp. nov.: a genetically homogeneous group composed of all strains of *Clostridium botulinum* toxin type G and some nontoxigenic strains previously identified as *Clostridium subterminale* or *Clostridium hastiforme*. International Journal of Systematic and Evolutionary Microbiology, 38(4), 375-381.

Tessari, E. N. C., Cardoso, A. L. S. P., Kanashiro, A. M. I., Stoppa, G. F. Z., Luciano, R. L., and de Castro, A. G. M. 2014. Analysis of the presence of *Clostridium perfringens* in feed and raw material used in poultry production. Food and Nutrition Sciences, 2014.

Timbermont, L., Lanckriet, A., Gholamiandehkordi, A.R., Pasmans, F., Martel, A., Haesebrouck, F., Ducatelle, R. and van Immerseel, F. 2009. Origin of *Clostridium perfringens* isolates determines the ability to induce necrotic enteritis in broilers. Comparative Immunology, Microbiology and Infectious Diseases, 32(6), 503-512.

1 Table 4.1 Methods to score coccidiosis lesions in broilers.

Score	*Eimeria tenella*	*Eimeria acervulina*	*Eimeria maxima*
0	No gross lesions	No gross lesions	No gross lesions
1	Very few scattered petechiae on cecal wall; no thickening of cecal walls; normal cecal contents present	Scattered, white plaque-like lesions containing developing oocysts. Maximum of 5 lesions per cm2	Small red petechiae may appear on the serosal side of the mid-intestine. No thickening of the intestine, though small amounts of orange mucus may be present.
2	Lesions more numerous with noticeable blood in the cecal contents; cecal wall somewhat thickened; normal cecal contents present	Lesions are much closer together, but not coalescent; no thickening of the intestinal walls; digestive tract contents are normal	Serosal surface may be speckled with numerous red petechiae; intestine may be filled with orange mucus; little or no ballooning of the intestine; thickening of the wall.
3	Large amounts of blood or cecal cores present; cecal walls greatly thickened; little, if any, fecal contents in the ceca.	Lesions are numerous enough to cause coalescence with reduction in lesion size and give the intestine a coated appearance. The intestinal wall is thickened, and the contents are watery.	Intestinal wall is ballooned and thickened. The mucosal surface is roughened; intestinal contents filled with pinpoint blood clots and mucus.
4	Cecal wall greatly distended with blood or large caseous cores; fecal debris lacking or included in cores.	The mucosal wall is greyish with colonies completely coalescent. The intestinal wall is very much thickened, and the intestine is filled with a creamy exudate	The intestinal wall may be ballooned for most of its length; contains numerous blood clots and digested red blood cells giving a characteristic color and putrid odor; the wall is greatly thickened;
Evaluation Site	**Ceca**	**Duodenum**	**Jejunum**

2 Source: Johnson and Reid (1970).

Table 4.2 Identification tests for the presumptive Clostridial spp. isolated from the feed samples collected.

Feed Mill	Origin of Sample	Morphology	Gram Stain (+/-)	Spore Forming	Lecithinase (+/-)	Hemolysis (α^1, β^2)	RapID™ ANA II Test (Probability)	16S Gene Sequencing[3]	Aerobic Growth
A	Post Mixing	Rods	+	Yes	+	double zone β	*C. perfringens* (> 99.9%)	+	No
A	Post Cooling	Rods	+	Yes	+	double zone β	--	+	Yes
A	Loadout	Rods	+	Yes	+	double zone β	--	+	Yes
B	Post Mixing	Rods	+	No	-	α	--	-	No
B	Post Pelleting	Rods	+	Yes	+	β	--	-	No
B	Post Pelleting	Rods	+	Yes	+	double zone β	*C. subterminale* (63.3%)	+	No
B	Post Pelleting	Rods	+	Yes	+	β	--	-	No
B	Post Cooling	Rods	+	Yes	+	α	--	+	No
C	CGM[4]	Rods	+	No	+	β	*C. innocuum* (>99%)	-	Yes
C	CGM[4]	Rods	+	No	+	β	*C. innocuum* (>99%)	-	Yes
C	Post Pelleting	Rods	+	No	+	α	*C. subterminale* (63.3%)	-	No
D	PBM[5]	Rods	-	No	+	α	--	-	Yes
D	PBM[5]	Rods	+	Yes	+	α	--	-	Yes
D	Post Mixing	Rods	+	Yes	+	double zone β	*C. perfringens* (>99.9%)	+	No
D	Post Cooling	Rods	+	No	+	α	*C. innocuum* (74.48%)	+	No
E	Corn Meal	Rods	+	Yes	+	α	--	-	No
E	Post Mixing	Rods	+	Yes	+	α	--	-	Yes
E	Post Pelleting	Rods	+	Yes	-	α	--	+	No
E	Post Pelleting	Rods	+	Yes	+	α	--	-	No
E	Post Cooling	Rods	+	No	+	α	*C. subterminale* (98%)	+	Yes
E	Loadout	Rods	+	Yes	+	α	--	+	Yes

α^1= Bacterial colonies displaying alpha hemolysis (greenish discoloration) on sheep blood agar.
β^2= Bacterial colonies displaying beta hemolysis (clearing of agar around colony) on sheep blood agar.
[3]= Positive sign (+) indicates isolates sent for gene sequencing; negative sign (-) indicates isolates not sent for gene sequencing
CGM[4]= Ingredient Corn gluten meal

PBM[5]= Ingredient Poultry by-product meal
-- = Test not performed.

Table 4.3 16S rRNA gene sequencing results.

Feed Mill	Origin of Sample	Classification by 16S Sequencing (% of Identity)[1]	Used in Live Bird Challenge
A	Post mixing	*Clostridium perfringens* (99%)	No
A	Post Cooling	*Bacillus proteolyticus* (99%)	No
A	Loadout	*Bacillus proteolyticus* (100%)	Yes
B	Post Pelleting	*Clostridium argentinense* (99%)	No
B	Post Cooling	*Clostridium argentinense* (99%)	No
D	Post mixing	*Clostridium perfringens* (99%)	Yes
D	Post Cooling	*Clostridium argentinense* (98%)	No
E	Post Pelleting	*Clostridium argentinense* (98%)	No
E	Post Cooling	*Clostridium argentinense* (98%)	No
E	Loadout	*Clostridium argentinense* (98%)	No

(% of Identity)[1]= Identity between isolate sent for 16S gene sequencing and the 16S Microbial Sequences Database

Table 4.4 Growth performance[1] of broilers challenged with *Eimeria* spp. and *Clostridium perfringens* or *Bacillus proteolyticus*.

Treatment	BW[4] Gain (Kg/10 birds)			FC[5] (Kg/10 birds)			FCR[6] (kg/kg)			Mortality (%)
	0 - 14	**14 - 28**	**0 - 28**	**0 - 14**	**14 - 28**	**0 - 28**	**0 - 14**	**14 - 28**	**0 - 28**	**0 - 28**
Unchallenged Control[2]	3.88 ± 0.06	9.02 ± 0.33	12.90 ± 0.27	5.06	15.22	20.28	1.3041	1.6874	1.5721	0
Cocci challenged Control[3]	3.76 ± 0.21	9.12 ± 0.45	12.88 ± 0.66	5.44	14.74	20.18	1.4468	1.6162	1.5668	0
Challenged (*C. perfringens*)	4.32 ± 0.03	9.78 ± 0.04	14.10 ± 0.14	5.72	15.68	21.40	1.3241	1.6033	1.5177	0
Challenged (*B. proteolyticus*)	3.80 ± 0.15	8.82 ± 0.10	12.62 ± 0.16	4.30	14.30	18.60	1.1316	1.6213	1.4739	0
P-value	0.193	0.455	0.317	-	-	-	0.083	0.835	0.654	-
Standard Error	0.081	0.201	0.259	-	-	-	0.061	0.073	0.063	-

[1] Values presented per 10 birds in a single cage per treatment.
[2] Unchallenged for *Eimeria* spp. or *C. perfringens*/*B. proteolyticus*.
[3] Challenged with *Eimeria* spp. only, not *C. perfringens*/*B. proteolyticus* challenge.
[4] Body weight gain expressed in Kg/10 birds ± Standard deviation.
[5] Feed consumption.
[6] Feed conversion ratio.

Table 4.5 Necrotic enteritis (NE) lesions of broilers challenged with *Eimeria* spp. and *Clostridium perfringens* or *Bacillus proteolyticus*.

Treatment	No. Birds	Deaths	Lesion Scores[3]				
			0	1	2	3	4
Unchallenged Control[1]	10	0	9	1	—	—	—
Cocci challenged Control[2]	10	0	9	1	—	—	—
Challenged (*C. perfringens*)	10	0	6	4	—	—	—
Challenged (*B. proteolyticus*)	10	0	9	1	—	—	—
P-value	-	-	0.158	0.158	-	-	-
Standard Error	-	-	0.433	0.433	-	-	-

[1] Unchallenged for *Eimeria* spp. or *C. perfringens*/*B. proteolyticus*.
[2] Challenged with *Eimeria* spp. only, not *C. perfringens*/*B. proteolyticus* challenge.
[3] NE lesions displayed by birds from each treatment using a NE scale described by Prescott et al., (1978).

Chapter 5.0 Summary and Conclusion

The projects presented in this thesis provides information regarding the bacterial contamination that exists in animal feeds collected from four commercial feed mills and the research feed mill from Auburn University. The contamination of animal feeds with pathogenic bacteria can occur from multiple environmental sources that harbor these pathogens, like the harvest of the grains (which may become contaminated from bacteria living in dust or soil carried through wind, rain or mechanical harvest), processing at the feed mill, transportation to the farms or during the storage of the feeds before feeding the animals (Maciorowski et al., 2006). Considering that the farm animals can acquire these bacterial pathogens by ingestion, the contamination of the animal feeds represents a risk of exposure, and these pathogenic bacteria may cause clinical or subclinical effects in the farm animals which could lead to a poor live performance and consequently to economic losses (Tessari *et al*., 2014). Additional concerns other than economic losses exist if the animals are contaminated with foodborne pathogens that may produce illness in humans like *Salmonella*, *Escherichia coli*, *Clostridium perfringens* or *Clostridium botulinum*. Therefore, the microbiological evaluation of the feed ingredients and animal feeds is necessary to determine if they represent a source of contamination to farm animals and potentially to humans.

In our first experiment (chapter 3.0) we were able to determine that the most contaminated feed ingredients among the five feed mills sampled were peanut meal and corn gluten meal for Clostridial spp. (CSC) and peanut meal and corn meal for *E. coli* (ECC). At the same time, we were able to determine that the least contaminated feed ingredients were soybean meal and distillers dried grains with solubles, for both CSC and ECC. For contamination of the mixed feeds during the manufacturing process (four stages: post mixing, post pelleting, post cooling and

loadout) at each feed mill we were able to determine that CSC contamination was the same in all stages of the feed manufacturing process. We were able to determine that contamination with ECC during the manufacturing process was higher at the post mixing stage, and that it was significantly reduced after the pelleting process. Recontamination of the mixed feed with ECC after the pelleting process was observed in 2/5 feed mills studied, therefore the pelleting process can't be used as a guarantee of *E. coli* free animal feed. We were not able to recover any *Salmonella* during this project, but an important result from this study was the confirmation of the presence of *Clostridium perfringens* in mixed animal feeds. This bacterium is implicit causing necrotic enteritis and gas gangrene in birds and mammal species (Hafez et al., 2011; Keyburn et al., 2008; Niilo, 1980). Finally, we determined the other bacterial species present in the mixed feeds that could represent a potential risk for disease transmission to farm animals.

The bacterial classification of the unknown species found in feeds is shown in our second experiment (chapter 4.0). We were able to identify the bacteria *Clostridium argentinense*, *Clostridium perfringens* and *Bacillus proteolyticus*. The pathogenic potential of the isolated *C. perfringens* and *B. proteolyticus* was evaluated through a necrotic enteritis model in broilers. That study showed that *B. proteolyticus* was not able to induce to disease and that the strain of *C. perfringens* evaluated was only able to induce to mild necrotic enteritis lesions using the scale described by Prescott et al. (1978). Further detail for a revaluation of the pathogenicity of these bacteria is discussed in chapter 4.0. For *C. argentinense* no live animal trial to determine pathogenicity was performed due to the insufficient information collected about the isolates and their ability to produce a neuroparalytic toxin that can cause botulism (Suen et al., 1988). Once confirmed that the *C. argentinense* isolates originally found in the animal feeds does not produce

the neurotoxin, an experimental trial with adequate safety and bio secure measurements will be performed to determine their pathogenic potential.

References:

Hafez, H. M. 2011. Enteric diseases of poultry with special attention to *Clostridium perfringens*. Pakistan Veterinary Journal, 31(3), 175-184.

Keyburn, A.L., Boyce, J.D., Vaz, P., Bannam, T.L., Ford, M.E., Parker, D., Di Rubbo, A., Rood, J.I. and Moore, R.J. 2008. *netB*, a new toxin that is associated with avian necrotic enteritis caused by *Clostridium perfringens*. PLOS Pathogens, 4(2): e26.

Maciorowski, K. G., Herrera, P., Kundinger, M. M., and Ricke, S. C. 2006. Animal feed production and contamination by foodborne *Salmonella*. Journal für Verbraucherschutz und Lebensmittelsicherheit, 1(3), 197-209.

Niilo, L. 1980. *Clostridium perfringens* in animal disease: a review of current knowledge. The Canadian Veterinary Journal, 21(5), 141.

Prescott, J. F., Sivendra, R., and Barnum, D. A. 1978. The use of bacitracin in the prevention and treatment of experimentally induced necrotic enteritis in the chicken. The Canadian Veterinary Journal, 19(7), 181.

Suen, J. C., Hatheway, C. L., Steigerwalt, A. G., and Brenner, D. J. 1988. *Clostridium argentinense* sp. nov.: a genetically homogeneous group composed of all strains of *Clostridium botulinum* toxin type G and some nontoxigenic strains previously identified as *Clostridium subterminale* or *Clostridium hastiforme*. International Journal of Systematic and Evolutionary Microbiology, 38(4), 375-381.

Tessari, E. N. C., Cardoso, A. L. S. P., Kanashiro, A. M. I., Stoppa, G. F. Z., Luciano, R. L., and de Castro, A. G. M. 2014. Analysis of the presence of *Clostridium perfringens* in feed and raw material used in poultry production. Food and Nutrition Sciences, 2014.