Investigation of the presence and seasonal prevalence of *Salmonella* spp., *Salmonella* Typhimurium, and its monophasic variant I 4,5,12:i:- in United States swine feed mills

by

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Abstract

Salmonella is an important pathogen of public health concern. Each year, *Salmonella* costs the food industry approximately $2.3 billion. In recent years, the number of cases of *Salmonella* linked to pork products has also increased in the United States (US). Although pork has the lowest association with human foodborne illness when compared to beef and chicken, it is the most consumed meat in the world. Therefore, *Salmonella* is a significant food safety concern for the American swine industry. This pathogen can be present along all the food production chain from farm to fork and recent studies reported the isolation of *Salmonella enterica* serovar Typhimurium (ST) and its monophasic variant 4,[5],12:i:- (STM) in feed and feed ingredients. The occurrence of these pathogens in the pre-harvest environment can translate to entry and contamination of the human food chain. Nevertheless, little is known about *Salmonella* incidence and association with these types of environments. Hence, the purpose of this study was to investigate the presence and seasonal prevalence of *Salmonella* spp., ST and STM in selected feed mills, among the major US swine feed production areas. Eleven swine feed mills in eight different states were selected. Six mills produced only mash feed, while the other five facilities produced both mash and pelleted feed. Visits were conducted during fall 2016, early spring 2017 and summer 2017. Twelve environmental samples were collected within each facility and season, representative of the production flow, from receiving of ingredients to the finished product, including floor surfaces, equipment dust, workers' shoes, and finished feed. Samples were analyzed following the USDA-FSIS guidelines and culture positive samples were analyzed by PCR. A multiplex PCR assay was also performed to differentiate *Salmonella* Typhimurium and *Salmonella* I 4,[5],12:i:- from the other serotypes. Associations between mill,

season, mill type, sample site and *Salmonella* prevalence were analyzed using generalized linear mixed models ($P < 0.05$). From the 383 samples collected, 49 (12.8%) were identified as *Salmonella* spp.; two (5.1%) were identified from feed, while the other 47 (13.7%) originated from equipment or surfaces. Two samples were positive for ST and three for STM by multiplex PCR. Mill ($P = 0.003$) and season ($P = 0.006$) were statistically associated with the presence of *Salmonella*, with higher prevalence in fall and summer (13.2%) as compared to spring (3.6%). These findings demonstrate the seasonal prevalence of *Salmonella* spp., ST and STM in feed mills across the US, highlighting the potential role of the feed mill environment as a microbial entry route into the human food chain. The data presented can be also used as a tool to assist in the implementation of mitigation strategies for pre-harvest food safety.

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Dedication

I dedicate this work to my family that encouraged and supported me from the beginning and throughout my studies, to my friends who made this journey better and helped make everything possible, either close to me or far away, your support was essential.

Chapter 1 - Introduction

Salmonella is one of the most important foodborne pathogens worldwide, it is among the main causes of foodborne illness in the US (CDC, 2011). This pathogen is ubiquitous and can survive in multiple conditions in the environment (Podolak et al., 2010) but is primarily associated with animal hosts such as poultry, cattle and swine (Tauxe et al., 1991; Benenson et al., 1995) . It has over 2500 serotypes, despite of a few serotypes becoming more adapted to specific host species, for example *S.* Choleraesuis in swine, they all still have the potential to infect humans (FDA, 2013). Animals can become infected with foodborne pathogens through contaminated feed (Crump et al., 2002), among other vehicles (Maciorowsky et al., 2006). Feed can be contaminated during its production in the feed mills via contaminated ingredients and/or the environment and equipment (Jones and Richardson 2004). Animals consume contaminated feed and can then harbor the bacteria without manifesting clinical signs, as asymptomatic carriers, while still shedding the organisms on their feces, promoting a cycle of pathogen spread within the farms and herds (Rostagno and Callaway, 2012). Recently, *Salmonella* has been linked to feed borne outbreaks (Osterberg et al., 2006; Molla et al., 2010). Once the animals are harvested, the processing of the carcass into pork cuts can result in the contact of contaminated gastrointestinal contents from infected pigs to its carcass and the others around it, via fomites such as knives, processing tables, and plant workers, or through the contact with contaminated lymph nodes (Olsen et al., 2001; Vieira-Pinto et al., 2005; Swanenburg et al., 2001). The resulting contaminated pork products can then be sold to the final consumer and result in human illness if the pork product is not properly cooked to *Salmonella* inactivation temperatures or if

there is cross contamination between the raw pork and other food items or food preparing surfaces (Carrasco et al., 2012).

Two *Salmonella* serotypes are strongly associated to pork and pork products, *S.* Typhimurium and *S.* 4,5,12:i:-, both appear among the most commonly isolated serotypes from animal feed and human infections in the US (Li et al., 2012). These serotypes have been responsible for numerous cases and outbreaks traced back to pork and pork products in recent years worldwide (Andres Barranco et al., 2016; CDC 2015; Gossner et al., 2011; Houser et al., 2010; Moreno-Switt et al., 2009; Mossong et al., 2007; Norton et al., 2012Tavecchio et al., 2004)

The US government is focusing in prevention of *Salmonella* transmission to humans (Fowler, 2013), and one of the strategies is focusing on the pre-harvest level. Therefore, the data on *Salmonella* prevalence in feed mills can assist in better developing those strategies and better target resources to a more effective prevention strategy.

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Chapter 2 - Statement of the problem: Are feed and feed mills a potential *Salmonella* entry route in the human food chain?

The genus *Salmonella* has over 2500 serotypes, divided in two main species: *S. enterica* and *S. bongori*, with most of the serotypes belonging to the *enterica* species. *Salmonella enterica* is categorized into six subspecies: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI) (Brenner et al., 2000). *Salmonella* species and subspecies, in addition to the number of serotypes within a subspecies and their usual habitats are shown in Table 1.1. Serotyping is based on the presence of lipopolysaccharides on the cell surface (O antigens), cell wall flagellar proteins (H antigens, divided into phase-1 and phase-2), and capsular proteins (Vi antigen). Some serotypes lack one of the H antigen phases and are classified as monophasic. Lack of phase-2 flagellar antigens is the consequence of either the absence of the *flj*B gene or the lack of gene expression (Argüello et al., 2014). Modern nomenclature designates numbered antigens to classify different serotypes. The international community follows the antigenic formula containing (i) the subspecies (I through VI), (ii) the somatic O antigens followed by a colon, (iii) the flagellar H antigens of phase-1 followed by another colon and (iv) the flagellar H antigens of phase-2 (represented by a dash if not present) (Brenner et al., 2000). For example, *Salmonella enterica* I 4,5,12:i:1,2 is the antigenic formula for *Salmonella* Typhimurium.

Salmonella is one of the most important pathogens impacting the food industry, being the leading cause of bacterial foodborne disease in the United States (US) with approximately 1.2 million human illnesses, 48,000 hospitalizations, and 360 deaths per year (CDC, 2011). While the impact on human health alone is enormous, there is also substantial economic repercussions

of *Salmonella* outbreaks and associated recalls across the food industry, resulting in an economic burden of $2.3 billion in the US (CDC, 2011).

Salmonella is a resilient microorganism that can live in low water activity conditions and adapt to different temperatures (Podolak et al., 2010). This pathogen can survive outside the animal host and in the environment (Baer et al., 2013*).* Moreover, *Salmonella* can persist for long periods of time, adapt to suitable host environments, and be a transient member of the animal gastrointestinal population. Recent studies have demonstrated the role of pigs as *Salmonella* reservoir (De Knegt et al., 2015). For example, pigs can ingest or inhale the pathogen from the environment or through contaminated feed (Fedorka-Cray and Hogg, 1997) and carry it during transfer from the farrowing farm to the finishing farm or to the slaughter house (Kranker et al., 2003). Some strains can persist within the intestinal tract and/or lymph nodes of swine during the production stages without causing illnesses and be transferred to meat cuts during slaughter (Arguello et al., 2013; Rostagno et al., 2009; Rostagno and Callaway, 2012) via cross-contamination of gastrointestinal contents and the carcass, directly or through fomites such as slaughter line workers, knives, and equipment (Bertrand et al., 2010). Generally, asymptomatic carrier pigs can be diagnosed at slaughter through an enzyme-linked immunosorbent assay (ELISA) of the carcass meat juices (Alban et al., 2002)

Although pork has the lowest association with human foodborne illness, when compared to beef and chicken, it is the most consumed meat in the world (Delgado et al., 2001), and the US is the third largest swine producer and first exporter in the world (USDA, 2016.). From 2006 to 2015, the number of clinical cases of *Salmonella* linked to pork products increased (CDC, 2014) and studies show that approximatelly 9 % of the reported salmonellosis cases in the US are associated with pork and/or pork products (Dickson et al., 2015). Therefore, *Salmonella* has

become a food safety concern also for the American swine industry. Ensuring the safety of pork products is essential for producers to maintain animal and human health, and to continue serving export markets (Baer et al., 2013*).*

Several studies have estimated the level of *Salmonella* in feed as generally low, and historically no evidence of a direct link to animal or human illness has been demonstrated in the US (Burns et al., 2015; Cochrane et al., 2015; Davies et al., 2004; Molla et al., 2010). However, animal feed has been responsible for salmonellosis outbreaks in Sweden (Österberg et al., 2006) and Finland (Haggblom et al., 2009). Nevertheless, the importance of feed as a pathogen contamination source in pigs, the potential risk of transmission and survival in slaughter houses, and the possible infection for consumers has been highlighted as significant and potentially high in some risk assessment models *(*Rönnqvist et al., 2017; Österberg et al., 2006). In Finland, Rönnqvist et al. (2017) developed a model to estimate human *Salmonella* infections linked to contaminated animal feed, through the consumption of pork. They concluded that the proportion of human cases that resulted from eating contaminated pork was 14%, representing 5.3% of domestic human salmonellosis cases per year in that country.

Animal feed is known to be a vehicle of transmission of pathogens to animals (Burns et al., 2015; Molla et al., 2010); *Salmonella* can contaminate feed and lead to animal infection (Crump et al., 2002). After ingesting *Salmonella* contaminated feed, animals can become sub-clinically infected and transition to a carrier state (Rostagno and Callaway, 2012). Carrier animals do not show clinical signs of disease but harbor the bacteria and infect other animals (Hurd et al., 2001; Kranker et al., 2003). Houser et al. (2010) determined that isolates recovered from human samples, pork products, and infected pigs shared identical traits when analyzed with molecular techniques, supporting the hypothesis that contaminated animals can lead to

contaminated food, and ultimately to infected consumers (Crump et al., 2002; Wasyl and Hoszowski, 2012).

The risk of salmonellosis from feed is difficult to quantify due to inconsistent data, sampling constraints and a lack of epidemiological information (Crump et al., 2002; Jones, 2011). Surveillance programs for *Salmonella* in animal products and feed have been implemented in the US (AFSS – Animal Feed Safety System, Feed Contaminants Program from 2002-2006, and the *Salmonella* Assignment from 2007-2009) and in Europe (Swedish National *Salmonella* Control Programme) (Abrahantes et al., 2009; Li et al., 2012; Österberg et al., 2006). While most *Salmonella* in swine is transmitted through fecal-oral and environmental cross contamination, animal feed is still a potential source of *Salmonella* infections for animals (Carter et al., 2003; Davis et al., 2003; Kidd et al., 2002). Potentially, all *Salmonella* serotypes can cause illness in animals and/or humans, but historically only a few have been associated to a determined animal species. The Compliance Guide Sec. 690.800 by the FDA considers animal feed to be adulterated when the product is contaminated with a serotype known to be pathogenic to the species for which the feed is intended. Examples of serotypes associated with animal disease are *S*. Pullorum, *S*. Gallinarum, and/or *S*. Enteritidis in poultry, *S*. Choleraesuis in swine, *S*. Abortusovis in sheep, *S*. Abortusequi in equine, and *S*. Newport and/or *S*. Dublin in cattle (FDA, 2013). Thus, swine feed contaminated with *Salmonella* is only considered adulterated if containing *Salmonella* Choleraesuis. Despite *S*. Choleraesuis being rarely reported, it is responsible for severe human infection resulting in bacteremia, in addition to being responsible for swine paratyphoid syndrome, resulting in production losses (Jean et al., 2006).

A surveillance study conducted in the US from 2002 to 2009 reported that 12.5% of feed and feed ingredient samples collected from manufacturing facilities were contaminated with

Salmonella spp. (Li et al., 2012). While *Salmonella* spp. contamination in livestock feed is low (Li et al., 2012), it is important to understand locations of entry into the animal feed value chain. For example, raw ingredients can come in contact with foodborne pathogens during transportation and storage (Crump et al., 2002). Once the raw ingredients reach production facilities, microbial contamination can occur while unloading ingredient due to dust creation, pests, and/or during processing and handling of the products (Maciorowski et al., 2006; Whyte et al., 2003). Moreover, the microbial load in feed and feed mills can be affected by moisture and temperature (Davies and Wales, 2010), differences in temperature between processes, for example the cooling of pelleted feed, have the potential to favor water condensation and that can increase the water activity of feed, thus . Since *Salmonella* spp. has been identified as a potential biological hazard in many livestock feeds (Cochrane, 2016; Crump et al., 2002), understanding this pathogen's ecological niche and potential pre-harvest entry routes into the human food chain is critical. These observations support the importance to investigate pathogen presence and possible transmission sources from feed to fork.

Figure 1.2. depicts a typical feed mill process flow. Generally, feed ingredients (grains), premixes (minerals and vitamins), medications, and animal ingredients (such as animal fat) are the raw ingredients received by the mills. The unloading process generates a lot of dust and ingredients might spill onto the floor (Cochrane, 2016). Premixes are received in bags and stored in the warehouse. Animal fat is received in a separate inlet that leads to the storage tank. Once the raw ingredients reach production facilities, microbial contamination can occur while unloading ingredients due to dust creation, pests, and/or during processing and handling of the products (Maciorowski et al., 2006; Whyte et al., 2003). Binter et al. (2011) demonstrate that all the ingredients used to fabricate animal feed have some degree of *Salmonella* presence (both

animal and vegetable derived feed material). Moreover, microbial loads in feed and feed mills can be affected by moisture and temperature (Davies and Wales, 2010).While loading/unloading, truck drivers can get off the truck and walk around the receiving area, representing a potential vehicle for biological hazard spread (Cochrane, 2016; Fedorka-Cray and Hogg, 1997) with their shoes, as reported by Amass et al., (2000) and Otake et al., (2002). In addition to drivers, the truck can also carry and spread pathogens. Whyte et al. (2003) found that 57.1% of vehicles transporting feed were contaminated with *Salmonella*. Furthermore, the warehouse and control room are easily accessible from the manufacturing area where most of the equipment such as the mixer, pellet mill, and cooler are located.

Finished feed, also called complete feed, is the final product containing the complete nutrient requirement for the targeted animal. Two types of feeds are available commercially in the swine industry: mash (non-pelleted, meal-based) or pelleted feed, which goes through extra processing steps, such as conditioning and heating (pelleting). The latter is considered a kill step for pathogenic bacteria and viruses and it is expected to reduce bacteria counts in finished feed (Burns et al., 2015; Maciorowski et al., 2006). Mash feed does not go through a thermal step and therefore requires particular attention to ingredients and manufacturing practices to ensure product safety (Binter et al., 2011). Binter et al. (2011) found that the prevalence of *Salmonella* in pelleted (4.6-8.0%) feed is lower than in mash (20.5-64.0%) finished feed. Probable causes of pelleted feed contamination are the failure in achieving target temperature/time/moisture during processing (Burns et al., 2015; Davies and Wales, 2010; Jones et al., 1991), and post-pelleting recontamination (Wierup and Häggblom, 2010).

Limited safety practices, such as hazard analysis, standard operational procedures, and good manufacturing practices (Cochrane et al., 2016) have been implemented for animal feed

environments, even though these facilities have been recognized as potential source of pathogens (Podolack et al., 2010; Rostagno et al., 2012). Although research has prompted the development of prevention strategies to ensure feed mill biosecurity, Cochrane et al. (2016) list a few key points, such as hazards identification and evaluation, likelihood of cross-contamination by people, probability of environmental cross-contamination, mitigation of biological hazards, and assessment to determine the effectiveness of the biosecurity plan. Examples of present practices to prevent contamination in the mill include: Purchasing safe ingredients from trustworthy suppliers, reducing the amount of dust generated during operations, segregation between "clean" and "dirty" areas within a plant, pest control, sanitary transportation of ingredients and feed, moisture control in the final product, and the use of a validated "kill" step (Jones, F. T., 2011).

Since the isolation of *Salmonella* from feed and the feed mill environment is labor intensive, several studies have investigated the relation between *Enterobacteriaceae* and *Salmonella* presence. *Enterobacteriaceae* are often used as fecal contamination indicators, and they can be considered a sign of poor hygiene, flaws during processing, and cross-contamination in feed mills (Jones and Richardson, 2004). Facilities and sample sites with high *Enterobacteriaceae* counts were also *Salmonella* positive, therefore researchers have suggested their use as quality indicator organisms to evaluate potential *Salmonella* presence (Jones and Richardson, 2004).

Among the clinically and economically relevant *Salmonella* serotypes linked to pork products, *Salmonella enterica* Typhimurium (ST) and its monophasic variant *Salmonella enterica* 4,5,12 :i:- (STM) are frequently reported as being among the most common causes of human salmonellosis cases (Table 1.2). In particular ST has evolved to be one of the most important serotypes concerning foodborne illnesses and is one of the most commonly found

serotypes in human clinical samples (EFSA, 2015). In addition to a high prevalence, ST displays, in many cases, a multi-drug resistance pattern with resistance to aminopenicillins, phenicols, aminoglycosides, sulphonamides, and tetracyclines (Andrés-Barranco et al., 2016). The Centers for Disease Control and Prevention (CDC) has linked ST to several outbreaks in the past years, including tomatoes, peanut butter, ground beef, produce, live poultry, frozen feeder rodents, chicken salad, and pork products (CDC, 2015). Before the mid-1990s, a variant of ST started to be identified in Europe and had the uniqueness of exhibiting only one H flagellar antigen (phase-1), with the antigenic formula I 4,5,12:i:-, called monophasic (STM) (Echeita et al., 1999). One of the first isolates was obtained from chicken carcasses in Portugal around 1986/87 (Machado and Bernardo, 1990). Since 1995, the reported cases of STM have increased in the United States (Moreno Switt et al., 2009), and within recent years STM has been progressively implicated in human disease worldwide. In 2013, STM was the third most common serotype linked to pig, pork, and clinical isolates in Europe (EFSA, 2015), and one of the six most common serotypes isolated from foodborne illnesses in the US (Moreno Switt et al., 2009). STM has recently caused a number of foodborne outbreaks and a number of them were traced to contaminated pork or pork products. In 2006, STM was responsible for two outbreaks likely associated to pork products in Luxemburg, causing 24 hospitalizations and one death (Mossong et al., 2007), in the US, a large recall from whole roaster hogs contaminated with STM in Graham, Washignton, was followed by investigations that traced the source of contamination to a pork slaughter establishment, and the potential sources of were identified as the raw pork meat, the inadequate employee handwashing practices, and the poor cleaning conditions of the surfaces and utensils used (CDC, 2015). The monophasic variant STM can exhibit a multi-drug resistance pattern broader than ST, this serotype is a particular concern because of its known resistance to many

antimicrobials commonly used for salmonellosis treatment in humans, including netilmicin, tetracycline, chloramphenicol, gentamicin, kanamycin, ampicillin, cephalothin, sulfonamides, sulfamethoxazole-trimethoprim, amoxicillin-clavulanic acid, streptomycin, amikacin, and nalidixic acid (Argüello et al., 2014; Echeita et al., 1999; Moreno Switt et al., 2009; Wasyl and Hoszowski, 2012), which is very concerning for human health.

Feed has been proven to be a vehicle of introduction and spread of important pathogens, such as multi-drug resistant *S.* Typhimurium and *S.* 4,5,12:i:-, potentially leading to foodborne outbreaks worldwide (Hsieh et al., 2016; Österberg et al., 2006). The manufacturing and distribution of safe feed is the first step to prevent animal disease and ensure a safer food chain from feed-to-fork, thus protecting the final consumer, and since *Salmonella* contamination is a multifaceted problem for the pork industry, a better knowledge of its contributing factors in the swine feed chain can assist in documenting steps where to focus preventive and control strategies more efficiently (Binter et al., 2011).

Rapid molecular technique for *Salmonella* detection

Traditional methods for the detection of *Salmonella* are culture based and consist of a series of steps, including pre-enrichment, selective enrichment, selective media plating, biochemical tests, and serological confirmation (Soria, et al., 2013; Wilkins et al., 2010). Modern molecular PCR based detection methods tend to be less labor intensive and time consuming, therefore providing more rapid analysis results. Bacterial detection with PCR assays can have different genes used as targets, for example the pathogenicity island *ttr*R gene (Jensen et al., 2013; Schelin et al., 2013; Zhang et al., 2013a), the flagella protein *fli*C gene (Prendergast et al.,

2013; Yang et al., 2013), and the enterotoxin *stn* gene (Riyaz-Ul-Hassan et al., 2013). A common target used for *Salmonella* detection is the gene responsible for encoding the type III protein secretion system, which is the largest inner membrane component, *inv*A. This gene is also necessary for bacterial invasion and virulence (Worrall et al., 2010) and is commonly used in PCR assays targeting *Salmonella* due to its conserved region in the genome among different serotypes (Barletta et al., 2013; Cunningham et al., 2010; Garrido et al., 2013; Li et al., 2014; Timmons et al., 2013; Zhang et al., 2013b). As part of our research, we helped validate a PCR assay based on the amplification of the conserved region *inv*A (presented in Appendix B) to confirm the diagnosis of the feed and feed mill samples that were identified as *Salmonella* spp. positive based on phenotypical characteristic on selective media, biochemical tests and serological agglutination procedures.

Serological agglutination is one of the steps used for conventional serotyping, based on the reaction of antisera and cell surface antigens present in the bacteria. This test sometimes fails to identify monophasic variants, such as *S.* 4,5,12:i:-, that could be reported as something else or unclassified (Prendergast et al., 2012). Identification of specific serotypes by PCR can provide more trustworthy results than the traditional technique and is recommended by the European Food Safety Authority (EFSA) to confirm the lack of phase-2 flagellar antigens in samples where agglutination is inconclusive (EFSA, 2010). Several multiplex PCR assays were considered in our study to differentiate between ST and STM. The selected assay was by Prendergast et al., (2013), who developed a multiplex real-time PCR for the identification and differentiation of ST and STM from other *Salmonella* serotypes, as an alternative to traditional agglutination techniques and conventional PCR assays previously used by Tennant et al. (2010) and Barco et al. (2011), to save time and resources. The targeted genes selected for the multiplex real-time

PCR assay were *fli*C, *flj*B 1,2, and *fli*B/IS200. These targeted genes are specific to ST and used to differentiate ST and STM from other serotypes: the *fli*C was chosen to identify the phase-1 flagellar antigen "i" present in ST and STM, in addition to some other serotypes (Aberdeen, Kedougou, Kentucky, and Lagos), the fljB 1,2 gene encodes the phase-2 flagellar antigen "1,2" present in ST and other serotypes (Coeln, Haif, Heidelberg, Paratyphi, Saintpaul, and Stanley) but not in STM, and the *flj*B/IS200 gene was selected because it is part of a conserved insertion region between genes *fli*B and *fli*A found in ST and STM (Prendergast et al., 2013).

Present study

This study is unique because it focus on the environment of commercial swine feed mills in the United States, more specifically on the presence of *Salmonella* spp. in the material accumulated and present on the surfaces of selected equipment used for feed manufacturing within the feed mills, in addition to the presence of *Salmonella* spp. in the material accumulated on the floor surfaces of selected areas within a feed mill, and not solely on contamination of finished feed.

Chapter 3 of this thesis aimed to collect swine feed and feed mill environment samples in order to determine the presence of *Salmonella* spp. in selected United States commercial animal feed mills in the months of October and November of 2016, and to characterize the prevalence of the pathogen in relation to sampling sites and processing-associated risk factors, such as the mills, their location, and the type of feed manufactured. In Chapter 4 of this thesis the investigation of prevalence of *Salmonella* spp. in swine feed mills in the US is expanded and, continuing from the study developed in Chapter 3, more interest points are added. In addition to

Salmonella prevalence, this chapter has the objective of studying the seasonality distribution of *Salmonella* in feed mills comparing data collected during February and March (spring) and June and July (summer) to those already obtained during the fall in Chapter 3. Moreover, another objective was to investigate two important *Salmonella* serotypes in the pork industry, ST and STM, among the samples obtained from the same feed mills across the US.

Tables and figures

Table 2-1. *Salmonella* species and subspecies, number of serotypes per subspecies, and their usual habitat, adapted from Brenner et al., 2000.

Salmonella species	*Salmonella* subspecies	Number of serotypes	Usual habitat
S. enterica	*enterica* (I)	1454	Warm-blooded animals
S. enterica	*salamae* (II)	489	Cold-blooded animals and the environment
S. enterica	*arizonae* (IIIa)	94	Cold-blooded animals and the environment
S. enterica	*diarizonae* (IIIb)	324	Cold-blooded animals and the environment
S. enterica	*houtenae* (IV)	70	Cold-blooded animals and the environment
S. enterica	*indica* (VI)	12	Cold-blooded animals and the environment
S. bongori		20	Cold-blooded animals and the environment

Table 2-2. Number and incidence of laboratory-confirmed *Salmonella* infections caused by the top 20 *Salmonella* serotypes, adapted from Rank - FoodNet, 2012.

Rank (2012)	*Salmonella* serotype	N of cases	% of total *Salmonella* cases
1	Enteriditis	1,239	15.8
2	Typhimurium	922	11.8
3	Newport	907	11.6
4	Javiana	757	9.7
5	S. I4,[5],12:i:-	340	4.3
6	Muenchen	191	2.4
7	Bareilly	183	2.3
8	Montevideo	183	2.3
9	Heidelberg	177	2.3
10	Saintpaul	163	2.1
11	Infantis	152	1.9
12	S. I 13,23:b:-	125	1.6
13	Thompson	105	1.3
14	Braenderup	100	1.3
15	Oranienburg	99	1.3
16	Mississipi	90	1.1
17	Typhi	57	0.7
18	Berta	55	0.7
19	Hadar	51	0.7
20	Hartford	47	0.6
	Sub total	5,943	75.8
	All other serotyped isolates	1,126	14.4
	Not serotyped isolates	431	5.5
	Partially serotyped isolates	272	3.5
	Rough or nonmotile isolates	70	0.9
	Total	7,842	100

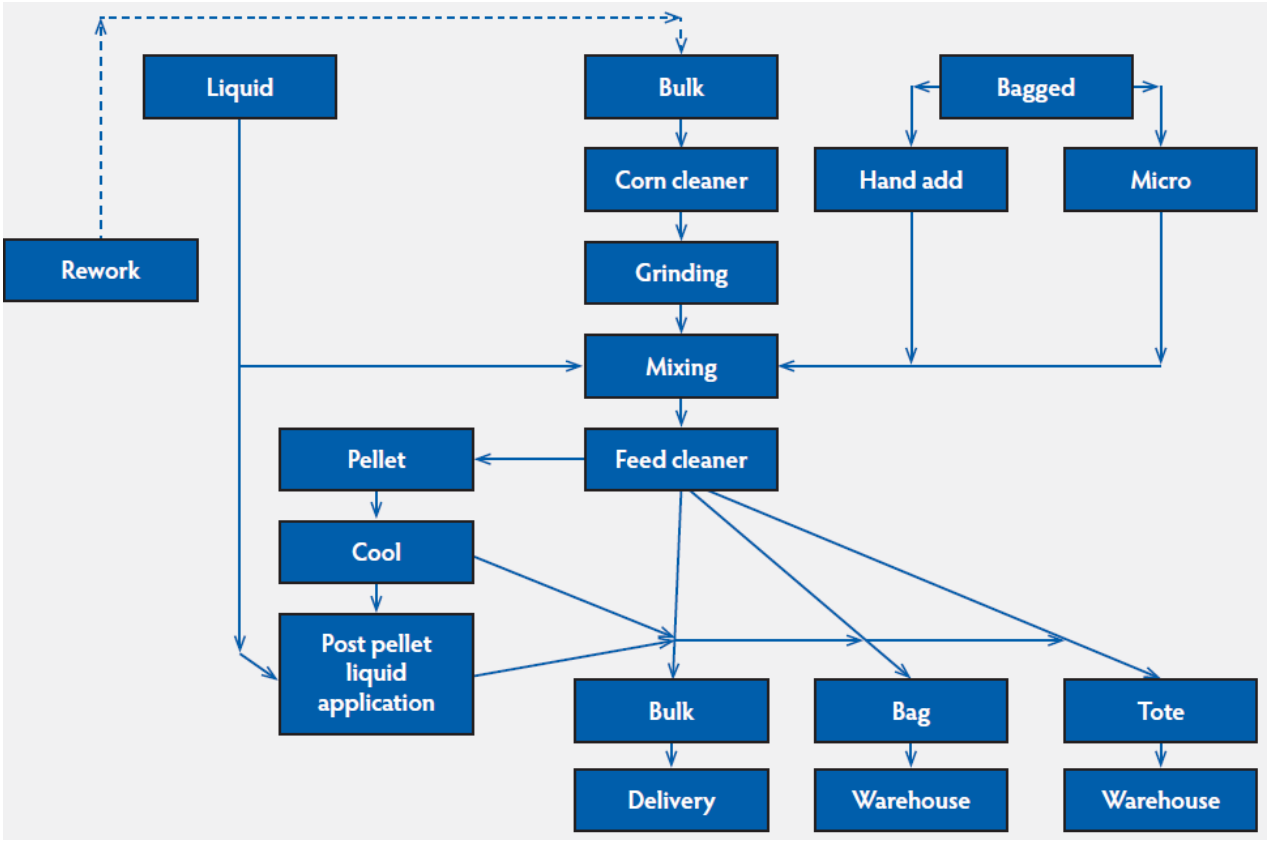

Figure 2-1. Example of a feed mill production flow for the manufacturing of pelleted feed (Cochrane et al., 2016)

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Chapter 3 - Evaluation of *Salmonella* presence in selected United States feed mills

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1. Summary

Salmonella is a pathogen of public health concern. Each year, *Salmonella* infections cost to the food industry approximately $2.3 billion and 33% of the reported cases are associated with beef, poultry or pork. Pathogen presence in feed mills can represent one of the many potential routes for entry and transmission into the food production chain. Nevertheless, little is known about *Salmonella* incidence and association with these type of environments. The objective of this study was to investigate *Salmonella* prevalence among environmental and feed samples in different commercial swine feed mills across the United States. Eleven facilities were selected in eight states and 12 sites were sampled within each feed mill. Samples were analyzed following the USDA-FSIS guidelines for isolation and identification of *Salmonella*. Positive isolates were further investigated by a PCR analysis targeting the *invA* gene to differentiate for *Salmonella enterica*. The total number of environmental samples collected was 237: 66% resulted culture positive and 13.1% were PCR positive. All sampled feed mills had at least one culture positive site, and following production flow, the number of positive samples decreased from ingredient receiving to final product. These preliminary results demonstrate the presence of *Salmonella* in selected United States feed mills and suggest their potential role as vehicle for pathogen transmission and spread into the food production chain.

2. Study objectives

The objectives of this study were to: 1) evaluate the presence of *Salmonella* in selected United States commercial animal feed mills; and 2) preliminarily characterize the prevalence of the pathogen in relation to sampling site and processing-associated risk factors.

3. Materials and methods

3.1 Swabbing method and sites

A diverse geographical pool of 11 feed manufacturing facilities, representative of the US swine production areas, were selected for this study. One location was identified each in Colorado, Illinois, Indiana, Minnesota and Oklahoma, whereas two were identified in Iowa, Kansas and North Carolina. Six mills produced only mash feed, while the other five facilities produced both mash and pelleted feed with average conditioning temperatures of 71°C for 45 sec, all mills manufacture swine feed commercially while some mills could manufacture customer-based formulas on a contract basis for other species, we did not collect that information.

Each mill was sampled once between the months of October and November 2016. Twelve sites within each facility were targeted for a total of 237 samples. The sites were selected considering production flow, people traffic and dust accumulation (Table 2.1). Samples were collected with a sterile sponge-stick pre-soaked in 10 mL of Buffered Peptone Water (3M, St Paul, MN). The surface of the receiving ingredient pit grating and floors from the receiving , manufacturing, warehouse and control/brake room areas were sampled in triplicates using a 10

cm × 10 cm sterile template. (Figure 2.1). Remaining sampling sites fat intake inlet, exterior of the pellet mill, finished product bin boot/product discharge, load-out auger and broom had one sample collected each visit(Figure 2.1). sponge samples were collected from shoes of production workers. Finished feed samples were also collected during each visit to the mills, a portion of feed (approximately 400g) was obtained from fresh feed manufactured that same day when the mills were visited. In mills producing mash feed, the finished feed sample was obtained after the mixing and before the loading out step, while in mills producing pelleted feed the finished feed sample was obtained after the pelleting and before the cooling step.

All samples were kept under chilled conditions and transported to the laboratory either by car or overnight shipping . Processing and testing of samples was conducted within 48 hours of sampling.

3.2 Culture-based analysis

The USDA-FSIS laboratory guidebook for the isolation and identification of *Salmonella* from meat, poultry, pasteurized eggs and catfish products, and carcass and environmental sponges was followed for culture-based analysis (FSIS, 2014). Samples were pre-enriched with of Buffered Peptone Water (BPW; BD Difco, Sparks, MD). A total of 60 mL of BPW were added to the environmental sponges following the USDA-FSIS guidebook and 450mL of BPW were added to 50g of finished feed samples, following the method described in Chapter 5 of the Bacteriological Analytical Manual (BAM, 2011), all samples were pre-enriched at 35 ± 2°C for 24 ± 2 h. After pre-enrichment, an aliquot (0.5mL and 0.1mL) was transferred to both Tetrathionate (BD Difco, Sparks, MD) and Rappaport-Vassiliadis, respectively (BD Difco, Sparks, MD) broths for selective enrichment and kept at 42°C for 24 h. Next, a 10µl sterile loop

was used to streak the enriched samples for isolation on both Brilliant Green Sulfa (BGS; BD Difco, Sparks, MD) and Xylose Lysine Tergitol 4 (XLT4; BD Difco, Sparks, MD) agar plates. Plates were incubated at 35 ± 2°C for 24-48h. Presumptive positive colonies were selected and one colony per plate was picked and analyzed with a combination of biochemical assays by stabbing and streaking the picked colony in both Lysine Iron Agar (LIA; BD Difco, Sparks, MD) and Triple Sugar Iron (TSI; BD Difco, Sparks, MD) agar slants. Presumptive positive samples were further investigated with a slide agglutination assay using a *Salmonella* polyvalent O antiserum test for groups A through G + iv following the manufacturer's instructions (BD Difco *Salmonella* O Antisera, Sparks, MD). A sterile needle was used to transfer the samples from the agar slants to an agglutination slide, containing a sterile 0.9% saline solution (BD Difco, Sparks, MD) and a polyvalent O antiserum droplet. Samples showing an agglutination reaction to the polyvalent O antiserum but not to the saline solution were considered agglutination positive.

3.3 Molecular-based analysis

Positive culture- based samples were further analyzed by real-time PCR. One colony from each agar plate was transferred directly and without any treatment to the PCR mixture. A protocol developed in our laboratory, that targets the invasion gene *inv*A present in all *Salmonella enterica* was followed (Bai et al., 2018). The PCR reaction was performed in a 25 µL final volume containing 10 µL of 1x iQ Multiplex Power mix (Bio-Rad, Hercules, CA), 1 µL of each primer (10 pM/µL), 0.5 µL probes (5 pM/µL), 2 µL DNA and nuclease-free water to make up the total reaction volume. Reactions were run on a CFX96 TouchTM Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using the following program: 10 min initial denaturation at 95°C followed by 45 cycles at 95°C for 15 sec and 62^{o}C for 50 sec. The 62°C

optimal annealing/extension temperature of the PCR assay was determined through a temperature gradient, in a two-step PCR protocol from which the annealing and extension stages were combined. For every experiment, a non-template control, a non-*Salmonella* control (*Escherichia coli* O157:H7 ATCC 43888) and four positive controls (*Salmonella* Newport ATCC 6962, *Salmonella* Typhimurium ATCC BAA-215, *Salmonella* Typhimurium monophasic variant 4, [5], 12:i:- CA RM 17 305 obtained from USDA ARS Albany CA, and *Salmonella* Typhimurium monophasic variant 4, [5],12:i:- NY FSL5-580 obtained from the Department of Food Science at Cornell University) were added. A sample was considered PCR positive when the Ct value was lower than 40.

3.4 Statistical analysis

Samples exhibiting typical colony phenotypes on selective agars (BGS and XLT4) and typical reactions in biochemical agar slants (LIA and TSI) compatible with *Salmonella* were considered culture positives (C+) and samples that were serotyped as *Salmonella* by the molecular assay were named PCR positive (PCR+). Descriptive statistics were computed to depict the number and percentage of test positive samples by sampling site and feed mill type. Sampling sites that were sampled in triplicates (surface of the receiving ingredient pit grating and floors from the receiving , manufacturing, warehouse and control/brake room areas) were denoted as positive if at least one of the three subsamples collected tested positive, the other sampling sites were sampled once, so a positive sample denoted the sampling site as positive. Percent positive samples was calculated as the number of test positive samples divided by the total number of samples collected by sampling site and by feed mill. Associations between explanatory variables (sampling site and mill type) with the prevalence of positive samples were

analyzed using generalized linear mixed models using Proc GLIMMIX in SAS 9.4 (SAS Institute Inc., Cary, NC, USA). A binary distribution, logit link, Laplace approximation and a *ridge*-stabilized *Newton*-Raphson algorithm were used. The outcome consisted of the presence of positive samples both by culture and molecular based analysis (dichotomous: positive vs negative). Independent variables included: mill ID (each individual mill received an ID consisting of a number from 1 to 11), state (state where the mill is located), mill type (divided into mills producing mash only or both mash and pelleted feed) and sample site (location within the mill that was analyzed).

An initial univariable screen was followed by a multivariable model if more than one fixed effect was significant in the univariable screen. Mean probabilities and their 95% confidence intervals were computed and significance was indicated by $P \leq 0.05$.

4. Results and discussion

For this study both C+ and PCR+ samples were considered: results from culture-based analysis gave an indication of *Salmonella* genus presence (family of *Enterobacteriaceae*), while molecular-based analysis provided specific information about *S. enterica* prevalence. Samples that were not C+ were analyzed by biochemical tests (API 20E, Biomeriux, Durham, NC). Results indicated that most of these isolates were either *Enterobacter* or *Citrobacter*. Several studies have shown that *Enterobacteriaceae* counts tend to be higher in *Salmonella* positive samples and that the presence of *Enterobacteriaceae* can be considered as an indicator of hygiene in feed mill production systems and a tool to assess the likelihood of *Salmonella* incidence (Jones and Richardson, 2004). Nevertheless, since results from the literature are

conflicting, our discussion concentrates only on the presence of *Salmonella (*both C+ and PCR+) in feed mill environments.

Table 2.2 shows the outcome from the univariable model: mill ID ($P < 0.001$), state ($P < 0.001$) and sampling site ($P = 0.0024$) were significantly associated with the presence of *Salmonella* spp., while mill type ($P = 0.3212$) was not.

Nevertheless, since most of the states selected for this study had only one feed mill visited, these two variables were considered confounded. The distribution of positive samples collected from feed mill facilities selected in this study is presented in Table 2.1. A total of 237 samples were tested: 157 (66.2 %) resulted *Salmonella* C+ and 19.8 % (n=31) were also PCR+. All feed mills analyzed in this study had at least one C+ *Salmonella* site (Table 2.1). The percentage of C+ samples was greater in sampling sites corresponding to worker shoes (92.5%), finished product bin boot (81.8%), ingredient pit grating (80.6%), and floor dust in receiving area (80.6%). Conversely, fat intake inlet (20%), exterior pellet mill (33.3%) and finished feed (41.7%) showed the lowest percentage of positive samples in the analyzed facilities. In our study we also observed that overall the number of C+ samples decreased from the initial processing steps towards the finished product, following feed production flow. As highlighter in Figure 2.1, the manufacturing process within the feed mill includes receiving, processing, storage-packaging, loading and delivery. Ingredients, people and cross contamination during production, load out and delivery were all identified as potential risks for microbial and viral introduction in feed mills (Cochrane et al., 2015). A biosecurity plan might offer an effective approach to reduce the likelihood of biological presence in feed mill manufacturing facilities, as well as microbial risk assessment and mitigation practices. Similar results of high pathogen presence in dust samples collected from manufacturing operations (33-65%), storage areas (10-27%) and worker

shoes (9-100%) were reported in a study that reviewed the practical measures to control *Salmonella* in animal feed (Jones and Richardson, 2004). This research highlighted the difficulty of detecting *Salmonella* in feed and the need to sample also dust and debris in feed manufacturing facilities to obtain a more sensitive indication of pathogen presence (Jones and Richardson, 2004). Based on these observations, in our study we selected sampling sites considering feed production flow, people traffic and dust accumulation. We also observed that the finished product bin boot had the highest number of C+ positive samples (81.1%) within the sampling sites in the production area. This equipment is in contact with the finished product before loading, therefore it was identified as a high-risk contamination point in our research; it might represent the primary entry point for *Salmonella* in the feed to fork chain.

Among the sampling sites that were not considered directly part of the production flow, worker shoes and broom had 95.2 and 63.6 % C+ samples, respectively. These results highlight the high likelihood of microbial transfer and cross-contamination within the facilities based on people movements (Cochrane et al., 2015). It was also observed that facilities manufacturing pelleted feeds had higher percentage of microbial presence in final products as compared to mash mills (Table 2.1). The unfiltered air introduced into the system to cool the feed after the pelleting step might represent the source of recontamination in this type of facility. Similar to our observations, another study on *Salmonella* contamination in US swine feed reported higher pathogen presence in pelleted commercial feed products as compared to on-farm mixed mash products (Davies et al., 2004).

All C+ positive samples were analyzed by quantitative PCR (qPCR) and 31 (19.8 %) were confirmed *S. enterica* (Table 2.1). Likewise, high pathogen presence was observed during the initial steps of production: ingredient pit grating (16.1%), floor dust in receiving area

(16.1%), floor dust in manufacturing area (19.4%), floor dust in brake/control room (16.1%), exterior pellet mill (16.7%) and finished product bin boot (18.2%). No PCR+ samples were detected form the load out-auger and fat intake inlet. Finish feed showed 8.8% PCR + samples. Within the sampling sites outside production flow, broom showed the greatest percentage of PCR + (27.3 %), followed by worker shoes (9.5%). As previously observed for C+ samples, microbial presence seems to be highly connected to people movement.

Since no data on weather conditions during sampling were recorded and no biosecurity plan details were obtained from the feed mill collaborators a longitudinal study might be needed to better define the influence of mill location and seasonality on pathogen prevalence. At this point we can only hypothesize that the facilities where the highest number of positive samples were detected did not have effective sanitation practices and/or cross-contamination occurred from incoming ingredients, employees, trucks or during other processing steps. Our results highlight the need of control measures in feed mill facilities to reduce the risk of Salmonella for both humans and animals. According to section 402 of the Federal Food, Drug, and Cosmetic Act (FD&C Act), FDA considers a feed to be adulterated if it is "contaminated with a *Salmonella* serotype that is considered pathogenic to the animal intended to consume the animal feed and the animal feed will not subsequently undergo a commercial heat step or other commercial process that will kill the *Salmonella*." For swine feed, only *Salmonella* Choleraesuis is considered to be adulterant. Nevertheless, certain animal serotypes, such as *Salmonella enterica* serovar Typhimurium and its monophasic variant serovar I 4,[5],12:i:-, that are not considered animal feed adulterants at present, can be carried by pigs without clinical signs and might enter the human food chain during harvesting operations (CDC, 2014).

5. Conclusion

Most peer-reviewed studies on *Salmonella* presence in commercial feed manufacturing facilities focus on final product, indicating the occurrence of pathogen contamination, but they lack information regarding pathogen environmental presence (Jones, 2011; Li et al., 2012; Molla et al., 2010). Contaminated feed can represent a vehicle for *Salmonella* transmission to animals and therefore increase pathogen likelihood to be introduced into the human food chain (Crump and Griffin 2002). Hence, understanding the mechanisms of contamination at the pre-harvest level in the livestock production is instrumental for a more thorough hazard analysis and biosecurity plan development: the goal is to prevent or reduce pathogen contamination in animal feed and decrease the possible entrance into the human food chain (Houser et al., 2010; Li et al., 2012).

To our knowledge, this is the first study to evaluate *Salmonella* presence in US feed mill environments. Our data indicates that feed manufacturing facilities can represent a port of entry for the pathogen into the food supply chain and that effective mitigation strategies are needed to identify contamination sources and reduce risk. Future studies exploring the seasonality, genetic relatedness, as well as serotyping and antibiotic resistance profiles of *Salmonella* isolates are warranted to fully understand the epidemiology, ecology and distribution of this pathogen in US feed mill environments.

Tables and figures

Table 3-1. Presence of *Salmonella* culture positive (C+) and PCR positive (PCR+) samples in feed mill facilities selected in this study

Pelleted feel mills[$]							**Mash feed mills**[$]						
Sampling site within the production flow	1	2	3	4	5	6	7	8	9	10	11	C+(%)	PCR+(%)
Ingredient pit gratin[2]	+++	--+	++-	+++	+++	+++	+	+++	-+-	+++	+-+	80.6	16.1
Floor dust in receiving[2]	+--	---	+++	+++	+++	+++	+	+++	+-+	+++	+++	80.6	16.1
Floor dust in manufacturing area[2]	+--	---	-+-	+++	---	+++	+	+++	++-	+++	-++	61.3	19.4
Floor dust in break or control room[2]	-+-	-++	+--	+++	-++	+--	+	+++	---	+++	+++	64.5	16.1
Floor dust in warehouse[1]	---	+-+	---	+-+	-++	++-	-	-+-	-++	+++	+++	54.8	3.2
Exterior of pellet mill	-	-	-	+	-	+	n/a	n/a	n/a	n/a	n/a	33.3	16.7
Finished product bin boot	-	+	+	+	+	+	+	+	-	+	+	81.8	18.2
Load-out auger	-	-	-	-	+	+	+	+	-	+	-	45.5	0.0
Finished feed	-	-	-	+	-	+	+	+	-	+	-	41.7	8.8
Sampling site outside the production flow													
Worker shoes[1]	++	-+	++	++	++	++	+	++	++	++	++	95.2	9.5
Broom	-	+	-	+	-	+	+	+	-	+	+	63.6	27.3
Fat intake inlet	-	-	-	*	-	+	-	-	-	+	-	20.0	0.0
										Total %		62.2	19.8

$ mills name, and location were substituted by number to protect collaborators privacy

[1]Left and right shoes swabbed

[2]Sites swabbed in three different location using a 10 cm x 10 cm template

*Site could not be sampled

n/a: Site not present in mash facilities

The % of PP and CP at the end of each row were calculated for sample sites

Highlighted + samples are PCR+

Table 3-2. Effects of variables on *Salmonella* spp. presence in the selected feed mills for this study.

Variable	P-value
Mill ID	<0.001
State	<0.001
Mill type	0.3212
Sampling site	0.0024

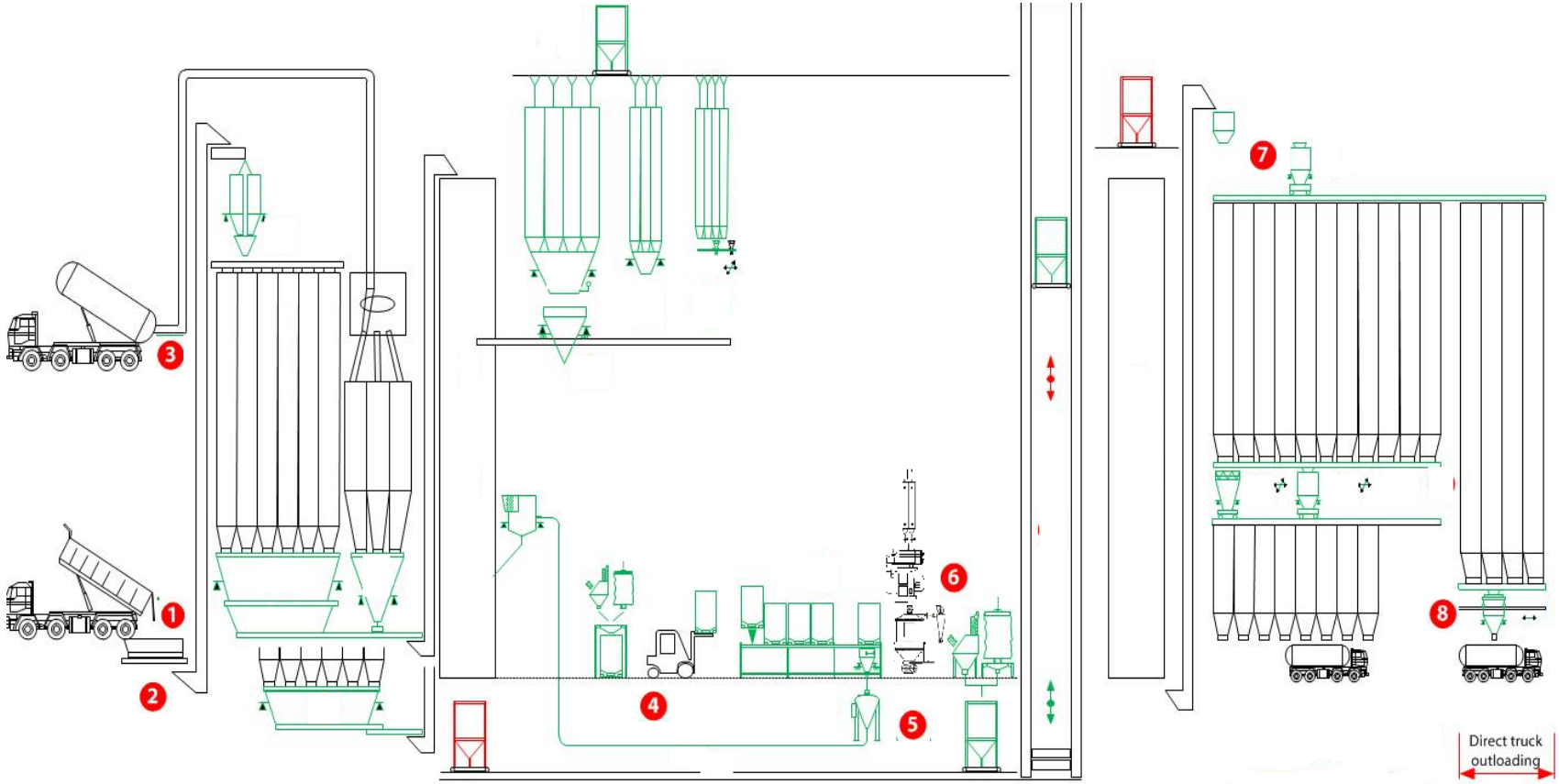

Figure 3-1. General layout of a feed mill production system with highlighted sampling sites, adapted from http://www.kse.nl/en/alfra/

1. Receiving ingredients pit grating
2. Receiving area
3. Fat intake inlet
4. Warehouse area
5. Manufacturing area
6. Pellet mill
7. Load-out auger
8. Discharge bin boot

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Chapter 4 - Seasonal presence of *Salmonella* spp., *Salmonella* Typhimurium and its monophasic variant serotype I 4,[5],12:i:- in selected United States feed mills

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1. Summary

The present study evaluated the seasonal prevalence and distribution of *Salmonella* spp., *Salmonella enterica* serovar Typhimurium (ST) and its monophasic variant 4,[5],12:i:- (STM) in selected feed mills across the United States. Eleven facilities were selected for this study and 12 sites were sampled within each mill during fall 2016, early spring 2017 and summer 2017. Samples were evaluated following the USDA-FSIS guidelines for *Salmonella* isolation and culture positive samples were analyzed by PCR. A multiplex real-time PCR was used to differentiate ST and STM from other serotypes. Associations between season, mill, and sample site with *Salmonella* presence were investigated using generalized linear mixed effects models. Both season ($P < 0.007$) and mill ($P < 0.005$) were significantly associated with *Salmonella* spp. presence. Fall months were associated with a higher *Salmonella* prevalence (13.2%) compared to early spring and summer. A total of 5 isolates, among the 383 samples, were serotyped as ST and STM . These two serotypes showed a similar seasonal presence throughout the study, being found during fall and summer seasons. These findings demonstrated the seasonal presence of *Salmonella* spp. in feed mills and the role of these environments as a potential pathogen entry route into the human food chain.

2. Study objectives

The objective of this study was to evaluate the seasonal prevalence and distribution of *Salmonella* spp., ST and STM in different feed mills across United States.

3. Materials and methods

3.1 Sample collection

Eleven feed mills distributed among eight states, representative of the main swine production areas within the US, were selected for this study (Figure 4.1). Each of the chosen mills supply feed to swine operations. Six mills produced only mash (non-pelleted, meal-based) feed, while the other five facilities produced both mash and pelleted feed. Within each feed manufacturing facility, twelve sampling sites were selected, taking into consideration production flow, people traffic, and dust accumulation (Table 4.1).

Samples were obtained by swabbing equipment and floor surfaces with a sterile sponge stick (3M, Saint Paul, MN) pre-soaked in Buffered Peptone Water (BPW) as previously described in Chapter 2 (Magossi et al., 2018). Floor surfaces (control room, receiving area, manufacturing and warehouse area and receiving ingredients pit grating) were swabbed using a 10 cm x 10 cm template in triplicate per visit. Worker shoes were swabbed over the entire bottom of each shoe, left and right. Feed manufacturing equipment (fat intake inlet, pellet mill, discharge bin boot, load-out auger, and broom) were swabbed once with no template, due to their shape. Finished feed samples were obtained after the pelleting (for pelleted feed) or after mixing (for mash feed) steps, approximately 400g were transferred to a sterile sampling bag within the mills before being transported to the lab for analysis. Samples were collected over three seasons: fall (October and November 2016), early spring (February and March 2017) and summer (June and July 2017), all samples, environmental and finished feed, were transported chilled by car or overnight shipping to the laboratory and were kept at 4°C for a maximum of 48h until analysis.

3.2 Culture and molecular based analysis

Samples were analyzed following the USDA-FSIS laboratory guidelines for the isolation and identification of *Salmonella* from meat, poultry, pasteurized eggs and catfish products, and carcass and environmental sponges (USDA, 2014). For feed samples, 50 g were used for analysis following the Bacterial Analytical Methods, chapter 5 (BAM, 2011). Sponges were pre-enriched with 60 mL of Buffered Peptone Water (BD Difco, Sparks, MD) at 35 ± 2°C for 24 ± 2 h, followed by a selective enrichment in both Tetrathionate broth (TT; BD Difco, Sparks, MD) and Rappaport-Vassiliadis broth (RV; BD Difco, Sparks, MD) at 42°C for 24 h, TT and RV enriched broths were then streaked for isolation on Brilliant Green Sulfa (BGS; BD Difco, Sparks, MD) and Xylose Lysine Tergitol 4 (XLT4; BD Difco, Sparks, MD) selective agars. Presumptive *Salmonella* positive samples, based on phenotypic appearance on BGS and XLT4 agar pates, were then submitted to biochemical tests in Lysine Iron Agar test (BD Difco, Sparks, MD) and Triple Sugar Iron Agar (BD Difco, Sparks, MD). Samples that tested positive based on culture were subjected to a real-time PCR assay adapted from Bai et al. (2018) targeting the *invA* gene present in *Salmonella enterica* and *Salmonella bongori*. Total reaction volume was 25µl (12.5 2X IQ Multiplex Power mix (Bio-Rad, Hercules, CA): 1µl of each primer (Bioresearch Technologies, Petaluma, CA), 0.5µl of probe (Bioresearch Technologies, Petaluma, CA) 10µl of nuclease-free molecular biology grade water (Integrated DNA Technologies, Coralville, IA), and a pick from a colony as the DNA template. PCR running conditions consisted of an initial denaturation step of 95°C for 10 min, followed by 40 cycles of 95°C for 15 seconds and 60°C for 50 seconds. Samples having a Ct value <40 were considered PCR positive (PCR+), and were classified as *Salmonella* spp.

3.3 Multiplex PCR

PCR+ isolates were further analyzed by a second multiplex PCR assay to differentiate ST and STM from other serotypes. The protocol described by Prendergast et al. (2013) was followed with minor modifications. A pick from a colony was transferred directly from an agar plate, with a pipet tip, to the PCR mixture without any treatment. The reaction was carried out in a final total volume of 25µl, containing 1µl of primer mix (0.4µM of each primer) (Bioresearch Technologies, Petaluma, CA), 0.5µl (0.2µM) of each probe (Bioresearch Technologies, Petaluma, CA), 12.5µl of 2X IQ Multiplex Power mix (Bio-Rad, Hercules, CA), and 10µl of nuclease-free molecular biology grade water (Integrated DNA Technologies, Coralville, IA). Three sets of primers and probes were used in the assay and the targeted genes were fliC (present in ST and STM), fljB 1,2 (present in ST), and fliB/IS200 (present in ST and STM) (Prendergast et al., 2013). The PCR was carried out in a CFX96 thermocycler (Bio-Rad, Hercules, CA), with an initial denaturation step of 94°C for 2 min, followed by 40 cycles of 95°C for 20 seconds and 60°C for 90 seconds. PCR reactions were considered positive when Ct values were ≤ 40. Samples were characterized as ST if expressing all three genes (fliC, fljB 1,2, and fliB/IS200) and STM if expressing both fliC and fliB/IS200 genes (Table 4.2).

3.4 Biochemical analysis

Samples classified as presumptive positive for *Salmonella* spp. based on colony characteristics on selective agar plates (BGS and XLT4) and biochemical agar slants (LIA and TSI) but showing a negative test result for the PCR assay targeting the invA gene present in *Salmonella* were selected for a biochemical screening to investigate their identity. One isolated colony from 41 out of 435 samples was tested with API 20E strips (Biomerieux, Durham, NC)

for the identification of gram negative *Enterobacteriaceae* according to the manufacturer instructions to determine the genus and species (is possible) of those samples.

Results of each biochemical test is classified as positive or negative and the combination of all the test results were interpreted with the online tool APIWEB, which determines the genus, species, and percentage of certainty of each samples.

3.5 Statistical analysis

Generalized linear mixed models were fitted in SAS 9.4 (SAS Institute Inc., Cary, NC, USA) using the glimmix procedure. Binary distribution, logit link, Laplace approximation, and ridge-stabilized Newton-Raphson algorithm were used. The outcome consisted of the presence of *Salmonella* spp. in environmental samples as determined by the PCR test (dichotomous: positive vs negative). Independent variables included: season (fall, spring, and summer), mill ID (each individual mill received an ID consisting of a number from 1 to 11), mill type (divided into mills producing only mash or both mash and pelleted feed), and sample site (numbered from 1 to 12, representing the sites in Table 4.1). When at least one of the subsamples (triplicates) of floor and worker shoes tested positive (PCR +) sample sites were considered positive. An initial univariable screen for the fixed effects of season, mill ID, mill type, and sample site was followed by a multivariable model if more than one fixed effect was significant ($P \leq 0.05$) in the univariable screen. Random effects considered for the univariable models were season, state, mill ID, mill type, month, and season. In an analysis where an effect was considered fixed, it was removed from the list of random effects. Mean probabilities and their 95% confidence intervals were computed (Table 4.1).

4. Results

A total of 383 environmental (n=344) and feed (n-39) samples were collected from eleven feed mills during three seasons. From the total isolates, 49 (12.8%) were *Salmonella* PCR +; two isolates (5.1%) were identified in feed, and 47 isolates (13.7%) from equipment and/or on surfaces. Based on the univariable models, season ($P < 0.007$) and mill ID ($P < 0.005$) were significantly associated with the presence of *Salmonella* spp., while mill type ($P > 0.952$) and sample site ($P > 0.170$) were not (Table 4.1). Samples collected during fall months had a significantly higher mean prevalence (13.2%) of *Salmonella* compared to samples collected during early spring (3.6%) or summer (6.7%) (Table 4.1). Nine of the total 11 feed mills had at least one *Salmonella* spp. PCR+ sample and the mean prevalence from all visits, determined by the univariable model, varied from 1.9 % to 37.5% across mills. Facilities 4 and 7 had the highest mean prevalence with 28.5% and 37.5%, respectively, whereas mills 9 and 11 had no *Salmonella* spp. positive samples (Table 4.1). As shown in Table 4.1, a higher mean prevalence of *Salmonella* spp. was observed in sites corresponding to the receiving area floor (20.9%), manufacturing area floor and receiving ingredients pit grating (14.7%), followed by control room floor and worker shoes (11.9%). Interaction between significant fixed effects (mill ID* season) were tested (P=0.999) and the random effects of month and state were considered confounded with season and mill ID, respectively. When an effect was considered fixed, it was removed from the list of random effects and the factors were analyzed independently. Therefore, mill ID ($P = 0.003$) and season ($P = 0.005$) were significantly associated with prevalence of *Salmonella* in our multivariable model.

A multiplex PCR was designed to identify ST and STM among the 49 PCR+ *Salmonella* isolates. A total of two ST and three STM were identified by the multiplex PCR. Both ST isolates originated from mill 5 and were recovered from the receiving area floor during summer. One STM isolate came from mill 1 and was identified in the control room floor during summer. The other two STM isolates were found in mill 10 during fall from the receiving ingredients pit grating and receiving area floor. These results suggest that feed mill contamination by ST and STM isolates may follow the same seasonal pattern as PCR + *Salmonella*, with higher prevalence during fall and summer. However, the relatively few samples evaluated in the current screening of feed mills makes this inconclusive. Additionally, the sample sites where ST and STM were recovered matched the highest percentage of PCR + samples.

Results from the API 20E biochemical tests are shown in Appendix E, where, among the 41 tested samples, 18 (44%) belonged to the genus *Enterobacter*, 12 (29%) *Citrobacter*, 3 (7%) *Klebisiella*, 2 (5%) *Cronobacter*, 2 (5%) *Pantoea*, and *Butiauxella*, *Pseudomonas*, *Proteus*, and *Escherichia coli* each had 1 (2%) positive sample.

5. Discussion

The Center for Veterinary Medicine (CVM) of the Food and Drug Administration (FDA) has implemented two programs for the surveillance of *Salmonella* in animal feed, under the Feed Contaminants Program, from 2002-2009, where samples from finished animal feed, feed ingredients, pet food, and pet treats were sampled and analyzed to determine *Salmonella* contamination on those animal food categories (Li et al., 2012). *Salmonella* have been shown to exhibit seasonal variation; higher prevalence in warmer months and lower in colder months

(D'Souza et al., 2004; Pangloli et al., 2008; Ravel et al., 2010). In our study, we observed a higher PCR + sample prevalence in the fall and summer seasons (Table 4.1), which is consistent with the findings from other studies (Ravel et al., 2010; Jahne et al., 2015). During warmer months, people tend to walk around the facility more often, go outside, and keep doors and windows open for air circulation. This behavior may leave the mill more susceptible to the entrance and spread of microorganisms. Conversely, during colder months people tend to remain inside and keep doors and windows closed. Other factors possibly contributing to the seasonality of bacterial contamination are the airborne transmission of *Salmonella* from high air particulate matter created during crops harvesting and fertilization (Jahne et al., 2015) associated with the use of swine manure as fertilizer that can be potentially contaminated with foodborne pathogens as *Salmonella* (Brooks et al., 2012) and the presence of insects, such as flies, that can also carry *Salmonella* (Panglioli et al., 2008) during the fall. That combination increases the availability and transmission of airborne pathogens into the mills.

Moreover, in our study, we observed a significant association between the feed mill ID and the prevalence of *Salmonella*. Differences in management, geographical location, hygiene practices, quality of incoming raw ingredients, volume of feed produced, number of workers, and time the facility has been operational are all important variables for pathogen presence as described by (Cochrane, 2016). Two different types of mills were included in our study: one producing mash feed only and the other both mash and pelleted feed, because the pelleting process is different than producing just mash feed. Pelleting introduces heat and moisture into feed, followed by a cooling step that is supposed to remove excessive moisture along with lowering the temperatures, therefore it is expected to be microbiologically safer. However, temperature differences occurring between fresh pelleted feed and cooler can lead to

condensation which increases the water activity of finished feeds and, consequently, permits the growth of pathogens such as *Salmonella* (Jones, 2011; Prendensen et al., 2008) Facilities were structurally different: extra equipment was present for the pelleting process (conditioner, extruder, pellet mill and cooler). No significant differences in *Salmonella* prevalence were observed between these two mill types, probably due to similar amount of dust accumulation and human flow as vehicles of microbial spread around the facility. Production flow and plant design might also play a role in preventing microbial introduction and recontamination of finished feeds (Whyte et al., 2003). Research studies have shown that raw grain ingredients and transporting trucks vehicle of contamination into the mill facilities (Binter et al., 2011; Fedorka-Cray and Hogg, 1997). As in our study, a high number of PCR+ samples were found in the receiving ingredient pit grating and receiving area floor. Additionally, birds and bird feces were found in some facilities, highlighting the vulnerability of these production environments to pests, wildlife, weather conditions, and human/vehicle traffic (Torres et al., 2011; Whyte et al., 2003). Because *Salmonella* can survive for long periods of time in dry and hostile environments, in our analysis we considered worker shoes as a potential microorganism reservoir (Table 4.1). Amass, et al. (2000) and Otake, et al. (2002) proved that shoes can carry biological hazards, like porcine reproductive virus and respiratory syndrome virus (PRRS). Therefore, workers` shoes can represent a vehicle for pathogen spread into and throughout the mill. Not surprisingly, in our study, the control room and manufacturing floor (areas with the highest human flow) showed a high percentage of PCR+ samples. Since our intent was to understand if a high prevalence of environmental contamination could lead to final product contamination as highlighted by Jones and Richardson (2004), finished feed was collected during each visit.

Among feed samples collected (n=39) only two (5.1%) were PCR+. These results are consistent with the FDA surveillance program findings from 2007-2009, where they observed that 5.6% of the total finished feeds from different animal categories, other than pet food, was contaminated with *Salmonella* spp.(Li et al., 2012). The prevalence of *Salmonella* spp. in finished feed observed in this study can be highly underestimating the real prevalence for a number of reasons. Primarily due to the difficulty in obtaining a representative amount of animal feed samples, which can be attributed to the nature of the manufacturing process (some mills can work 24h a day for seven days a week) (Davies, 2004; Häggblom, 2009). Nevertheless, contaminated feed still remains a potential source of salmonellosis cases and outbreaks in production animals (Osterberg et al., 2006; Molla et al., 201).

The two *Salmonella* positive finished feed samples came from mills 4 and 7 and both facilities had the highest mean prevalence of *Salmonella* among the mills we observed; 28.4 and 37.4 % respectively. Jones and Richardson (2004) highlighted how the dust in the environment can be a source of equipment and feed mill contamination of *Salmonella*, and this contributes to a persistent contamination of a mill (David and Wales, 2010). Therefore, the high *Salmonella* prevalence in feed mill environments can be logically connected with a greater risk of cross-contamination of finished feed.

The final goal of our study was to identify, among PCR + samples, the presence of ST and STM serotypes. These two serotypes seemed to show overall a similar seasonal presence throughout the study, as compared to S*almonella* spp., being found most commonly during fall and summer. ST and STM samples were recovered from sampling sites corresponding to the receiving of ingredients area, highlighting the potential risk of introduction of pathogens into the feed mills through contaminated ingredients. The exception was a STM positive sample obtained

from the control room floor, an area of high human traffic, which can have the potential to spread this pathogen within the mill via workers shoes. However, there were only five positive samples for these two serotypes, a low number to be analyzed in comparison with the total *Salmonella* positive samples observed. STM, along with ST, is one of the most commonly found serotypes in humans, swine and pork products in recent decades (Hauser et al., 2010; Moreno Switt et al., 2009). STM isolates have been shown to be resistant to many antibiotic drugs generally used to treat human patients. (Andres-Barranco et al., 2016)

The discrepancy between the number of presumptive positive samples, those displaying phenotypical characteristics of *Salmonella* spp. on selective media (BGS and XLT4) and biochemical agar slants (LIA and TSI), and the *Salmonella* PCR+ samples is relevant. This shows that it is necessary to serologically and molecularly confirm isolates that are positive on initial steps of *Salmonella* screening from environmental and feed samples. Additionally, only one colony with typical *Salmonella* characteristics was picked from each plate and that can be a source of false positives, since the selective media help prevent the growth of unwanted non-*Salmonella* bacteria, but it is not 100% effective and still allows the growth of those organisms.

The data gathered in this study shows the potential role of feed and feed mill environments as entry routes for *Salmonella spp.,* ST and STM into the human food chain. These observations should support the implementation of effective biosecurity plans and other preventative strategies for controlling *Salmonella* in feed mills. For a complete assessment of *Salmonella* prevalence in the US mills, further experiments will be necessary to extend the geographical range to more states and increase the number of mills participating in the study (more mills per state). A larger sample set would significantly improve the quality and predictive

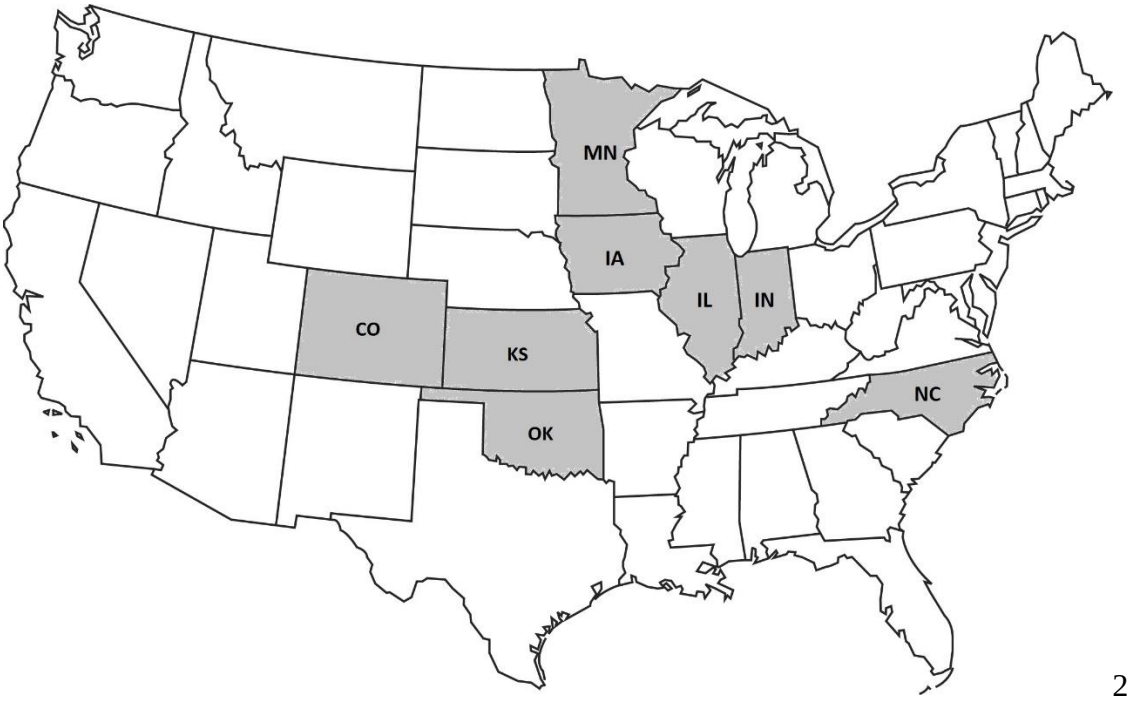
value of our current study. It is important to emphasize that the results obtained are a reflection of the study population of feed mills and may not represent other groups of mills.

6. Conclusions

Our study demonstrated the common presence of *Salmonella* in feed mills across the US. A seasonal pattern was observed with higher pathogen prevalence in fall and summer. A total of 5 ST and STM isolates were found among the 49 PCR+ samples, from the total 383 samples collected. Hygiene, management, production flow, and cross-contamination within a facility are all important factors previously linked with pathogen contamination in mills. We found that both the mill and the season were significantly associated with *Salmonella* prevalence. The sample sites selected in this study might not encompass all of the possible contamination sites within the mills. Only one finished feed sample was collected per visit, which could underestimate the true contamination status of the production facility. These findings contribute to a better understanding of *Salmonella* ecological niches in the animal feed processing environment. Antibiotic resistance patterns, genetic relatedness, and origin of *Salmonella* isolates should also be investigated to confirm pre-harvest microbial entry routes into the human food chain. and Moreover, it is important to emphasize that this study evaluated the *Salmonella* prevalence in selected mills across US, thus may not accurately depict contamination rates at the approximately total feed mills in the US.

Tables and figures

1



2

Figure 4-1. Map of the main swine production areas across the United Stated (highlighted in map 1), and the location where feed mills were selected for this study (map 2).

Table 4-1. Presence of *Salmonella* PCR + samples in feed mills by season, mill ID, mill type and sampling site

Variable	n[1]	Model-adjusted[2]		
		Mean prevalence (%)	95% CI (%)	*P* - value
Season				0.006
Fall 2016	25	13.2	5.1-29.7	
Spring 2017	9	3.6	1.1-11.0	
Summer 2017	15	6.7	2.3-17.9	
Mill ID				0.005
1	5	10.8	3.9-26.8	
2	1	1.9	0.2-13.6	
3	3	6.2	1.7-19.9	
4	11	28.4	14.0-49.1	
5	6	13.9	5.4-31.5	
6	2	3.9	0.9-16.1	
7	13	37.4	19.9-58.8	
8	3	6.7	1.8-21.4	
9	0	0.0	0.0-100	
10	5	11.9	4.1-29.6	
11	0	0.0	0.0-0.0	
Mill ID*season				0.999
Mill type				0.952
Mash	27	6.8	1.7-23.5	
Pelleted	22	7.3	1.8-25.6	
Sample site				0.170
1. Receiving ingredients pit grating	7	14.7	4.0-41.5	
2. Fat intake inlet	1	1.6	0.2-14.7	
3. Pellet mill	2	7.5	1.1-36.1	
4. Discharge bin boot	3	5.0	1.0-21.9	
5. Load-out auger	0	0.0	0.0-100	
6. Finished feed	2	3.0	0.5-16.6	
7. Control room floor	6	11.9	3.1-36.7	
8. Receiving area floor	9	20.9	6.3-50.7	
9. Manufacturing area floor	7	14.7	4.0-41.5	
10. Warehouse area floor	3	4.7	1.0-21.9	
11. Worker shoes	6	11.9	3.1-36.7	
12. Broom	3	5.0	1.0-21.9	
Total	49			

[1] Number of *Salmonella* positive (PCR+) samples per variable considered in this study.

[2]Model-adjusted prevalence estimates from univariable models evaluating the association between each variable with the presence of *Salmonella* spp.

Table 4-2. Targeted genes and primer sequences for the differentiation of *Salmonella* Typhimurium and S. I 4,5,12:i:- using a multiplex real-time PCR assay

Target	Description	Sequence (5`-3`)	Reference
*fli*C	Forward primer	CCC-CGC-TTA-CAG-GTG-GAC-TAC	O`Regan et al. (2008)
	Reverse primer	AGC-GGG-TTT-TCG-GTG-GTT-GT	
	Probe	CY5-TAA-AGC-CGC-ATT-GAC-AGC-AGC-AGG-TG-BHQ2	
*flj*B 1,2	Forward primer	TGT-TAC-TAT-TGG-CTT-TAC-TGG	Munoz et al. (2010)
	Reverse primer	CAG-CAG-GCA-TTG-TGG-TCT-TAG	
	Probe	JOE-CGC-CAG-CCG-CAA-GGG-TTA-CTG-TAC-BHQ1	
*Fli*B/IS200	Forward primer	GAT-CTG-TCG-ATG-ATT-CAT-CTT-CTG-AC	Prendergast et al. (2013)
	Reverse primer	AAC-GCT-TGT-CTT-CGG-TAT-TTG-G	
	Probe	FAM-TCG-GGT-GTG-CGC-TAA-GCT-CTT-TT-BHQ1	

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Chapter 5 - Future research

The results of this study demonstrate the presence of *Salmonella* spp., *Salmonella* Typhimurium, and its monophasic variant *S*. 4,5,12:i:- in finished swine feed and feed mill environments in the United States. Additionally, it was observed that *Salmonella* presence was associated with season and mill ID; however, it was not associated with the type of mill (mash or pelleted) or with the sample collection site within the mill. This research represents a first step to elucidate missing information on the ecology and transmission route of *Salmonella* in a feed-to-fork model.

Clinical foodborne salmonellosis outbreak cases have been previously linked to contaminated feed previously in other studies and risk assessment models. Future research should focus on the transmission of these pathogenic serotypes within the meat (pork) production chain: (i) from the feed mill environment to finished feed during production and transportation, (ii) from feed to pigs at the farm level, and (iii) from the pigs to pork products, during slaughter, and manufacturing, and until the consumer actually purchases and consumes pork products. Data regarding the transmission of *Salmonella* at pre-harvest level will be crucial to develop measures that effectively prevent and control *Salmonella* contamination in the human food chain, therefore reducing the economic and public health burden associated with this foodborne pathogen.

More broadly, a more accurate and comprehensive understanding of contamination scenarios within swine feed manufacturing would allow the envisioning of effective pathogen reduction technologies and more effective implementation of feed safety programs across the industry, potentially resulting in the reduction of the health and economic burden that is associated with *Salmonella*.

Appendix A - Sample sites within feed mills selected for the studies

Figure A-1. Receiving ingredients pit grating

Figure A-2. Fat intake inlet

Figure A-3. Pellet mill

Figure A-4. Discharge bin boot

Figure A-5. Load-out auger

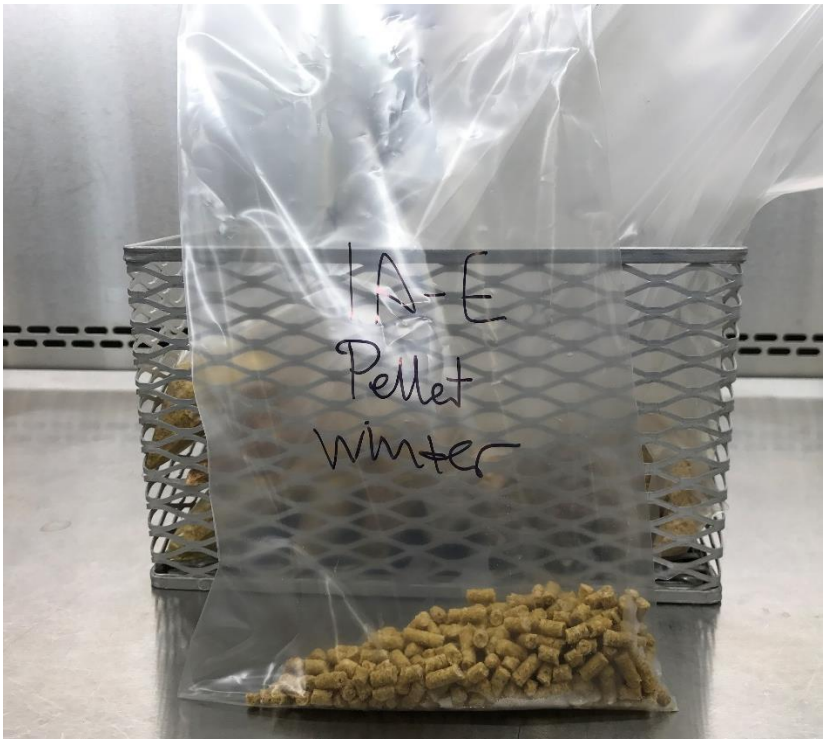

Figure A-6. Finished feed (pelleted)

Figure A-7. Control room floor

Figure A-8. Receiving area floor

Figure A-9. Manufacturing area floor

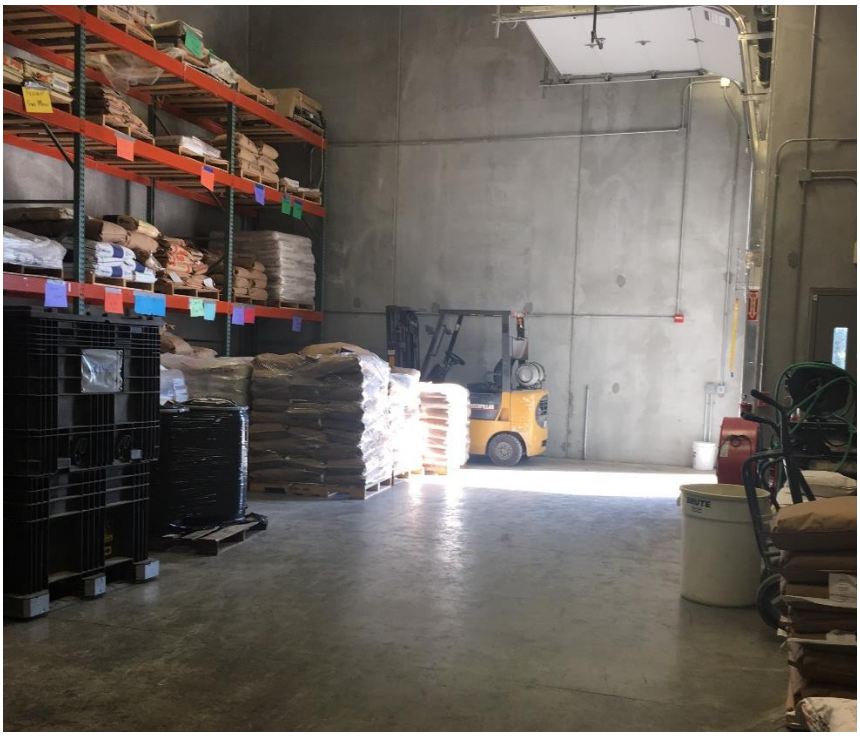

Figure A-10. Warehouse area floor

Appendix B - A multiplex real-time PCR assay, based on *inv*A and *pag*C genes, for the detection and quantification of *Salmonella* enterica from cattle lymph nodes

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1. Summary

Cattle lymph nodes can harbor *Salmonella* and potentially contaminate beef products. We have developed and validated a new real-time PCR (qPCR) assay for the detection and quantification of *Salmonella enterica* in cattle lymph nodes. The assay targets both the *inv*A and *pag*C genes, the most conserved molecular targets in *Salmonella enterica*. An 18S rRNA gene assay that amplifies from cattle and other animal species was also included as an internal control. Available DNA sequences for *inv*A, *pag*C and 18S rRNA genes were used for primer and probe selections. Three *Salmonella* serotypes, *S.* Typhimurium, *S.* Anatum, and *S.* Montevideo, were used to assess the assay's analytical sensitivity. Correlation coefficients of standard curves generated for each target and for all three serotypes were >99% and qPCR amplification efficiencies were between 93% and 110%. Assay sensitivity was also determined using standard curve data generated from *Salmonella*-negative cattle lymph nodes spiked with 10-fold dilutions of the three *Salmonella* serotypes. Assay specificity was determined using *Salmonella* culture method, and qPCR testing on 36 *Salmonella* strains representing 33 serotypes, 38 *Salmonella* strains of unknown serotypes, 252 *E. coli* strains representing 40 serogroups, and 31 other bacterial strains representing 18 different species. A collection of 647 cattle lymph node samples from steers procured from the Midwest region of the US were tested by the qPCR and compared to culture-method of detection. *Salmonella* prevalence by qPCR for pre-enriched and enriched lymph nodes was 19.8% (128/647) and 94.9% (614/647), respectively. A majority of qPCR positive pre-enriched samples (105/128) were at concentrations between 10^4 and 10^5 CFU/mL. Culture method detected *Salmonella* in 7.7% (50/647) and 80.7% (522/647) of pre- and post-enriched samples, respectively; 96.0% (48/50) of pre-enriched and 99.4% (519/522) of post-

enriched culture-positive samples were also positive by qPCR. More samples tested positive by qPCR than by culture method, indicating that the real-time PCR assay was more sensitive. Our data indicate that this triplex qPCR can be used to accurately detect and quantify *Salmonella enterica* strains from cattle lymph node samples. The assay may serve as a useful tool to monitor the prevalence of *Salmonella* in beef production systems.

2. Study objectives

The objectives of our study were to: 1) develop a duplex quantitative (real-time) PCR (qPCR) assay targeting the *inv*A and *pag*C genes; 2) validate the assay detecting and quantifying *Salmonella enterica* in cattle feces and lymph node samples collected from slaughter plants; and 3) test the versatility of the method for the detection of *Salmonella* strains isolated from feed and environmental samples from feed production facilities.

3. Materials and methods

3.1 Primers and probes

All available sequences of *inv*A and *pag*C genes from *Salmonella enterica*, and the 18S rRNA gene from different animal species were downloaded from the GenBank website (https://www.ncbi.nlm.nih.gov/genbank/), aligned and analyzed to identify conserved regions that could serve as potential targets. Primers and probes were selected using the online PCR design tool, Primer3 (http://bioinfo.ut.ee/primer3-0.4. 0/) (Untergasser et al., 2012) (Table 3.1). The *pag*C design was specific to *S*. enterica, but the *inv*A assay will detect both *S. enterica* and *S.*

bongori strains due to high identity of the gene (> 99%) from both *Salmonella* species. FAM, MAX (VIC-equivalent) and Cy5 channels were selected for *inv*A, *pag*C and 18S rRNA targets, respectively. All nine oligomers were checked for potential formation of secondary structures using the AutoDimer software (Vallone and Butler, 2004). Primers and probes were synthesized from Integrated DNA Technologies (Coralville, IA).

3.2. Assay optimization and standard analysis with pure cultures

Three *Salmonella* serotypes from our −80 °C storage collection, that include *S.* Typhimurium (ATCC 14028), *S.* Anatum (TX2006, C20) and *S.* Montevideo (TX 2006, C7), were streaked onto blood agar plates (BAPs, Remel, Lenexa, KS). A single colony of each strain was transferred into a 10 mL Luria-Bertani (LB; Becton, Dickinson Co., Sparks, MD) broth and incubated overnight at 37 °C. One hundred microliters of the overnight culture was then inoculated into 10 mL of LB broth and incubated at 37 °C until an absorbance of 0.4 at 600 nm was achieved (~2.5 h incubation and approximate cell concentration of 107–108 CFU/mL). Ten-fold serial dilutions in LB broth were prepared. Culture dilutions were also used for lymph node inoculations described below. Aliquots of 100 μL from 10−5, 10−6 and 10−7 dilutions were spread-plated onto BAPs to determine viable bacterial cell concentrations. One milliliter of culture from each dilution was also boiled for 10 min and centrifuged at 9300 g for 5 min; the supernatant was used as template for qPCR reactions. Each qPCR reaction was performed in a 20 μL total volume that contained 10 μL of 2× iQ Multiplex Powermix (Bio-Rad, Hercules, CA), 1 μL of each primer (10 pM/μL), 0.5 μL of each probe (10 pM/μL), 2 μL of DNA and 0.5 μL of nuclease-free water. Reactions were run on a CFX96 Touch Real-Time PCR Detection System (BioRad, Hercules CA) using the following assay running conditions: 10 min initial denaturation

at 95 °C followed by 45 cycles of 95 °C for 15 s and 62 °C for 50 s. The 62 °C optimal annealing/extension temperature of the PCR assay was determined through a temperature gradient assay in a two-step PCR protocol in which the annealing and extension stages were combined. All PCR reactions were performed in triplicate, and each standard curve was replicated for each serotype. Correlation coefficients and PCR amplification efficiencies were determined using the CFX Manager software (BioRad, Hercules CA).

3.3. Lymph node homogenates spiked with *Salmonella*

Ten-fold serial dilutions of the three *Salmonella* serotype (*S.* Typhimurium, *S.* Anatum and *S.* Montevideo) cultures prepared in Section 2.2 were also used for replicated inoculations of *Salmonella*-negative lymph nodes. *Salmonella*-negative lymph nodes previously screened by PCR were used for the three *Salmonella* serotype strain inoculations with replication. The *Salmonella* negative status of the lymph nodes were confirmed by enrichment steps described below followed by PCR testing. Three hundred microliters of each dilution were added to a 2.7 mL lymph node homogenate (described in Section 2.5 below) for a final volume of 3 mL. The homogenate without inoculum addition was considered as a negative control. One milliliter of each *Salmonella*-lymph node homogenate mix (pre-enrichment) was kept separately for DNA extraction using a GeneClean kit (MP Biomedicals, Santa Ana, CA). Extracted DNA was used to generate qPCR standard curves to determine the limit of detection for each serotype (Table 3.2).

The remaining homogenate mix was then incubated at 25 °C for 2 h then at 42 °C for 12 h. Following incubation, 1 mL of enriched homogenate was subjected to immunomagnetic separation (IMS) with anti-*Salmonella* IMS beads (Dynal Inc., New Hyde Park, NY). One hundred microliters of phosphate buffered saline (PBS, Sigma-Aldrich, St. Louis, MO) was

added to the final IMS step. The bead suspension was then transferred into 3 mL Rappaport-Vassiliadis broth (RV, Becton, Dickinson, Sparks, MD) and incubated at 42 °C for 18–20 h. One milliliter of enriched RV broth (post-enrichment) was then subjected to GeneClean DNA extraction and qPCR as previously described.

3.4. Assay specificity by real-time PCR

Specificity of the assay was tested with 36 *Salmonella* strains representing 33 *Salmonella* serotypes, 10 additional *Salmonella* isolates from feed and 28 isolates from environments of different animal feed production facilities; 252 *E. coli* strains representing 40 O-serogroups, and 31 strains belonging to 18 other bacterial species were also included in the assay specificity evaluation. *Salmonella* serotypes (no. of strains) were Typhimium (3), Bareilly (1), Derby (1), Enteritidis (2), Infantis (1), Reading (1), Anatum (1), Mbandaka (1), Montevideo (1), Kentucky (1), Newport (1), Orion var. 15+ 34+ (1), Uganda (1), Uganda var. 15+ (1), Thompson (1), Meleagridis (1), Muenchen (1), Bredeney (1), Oranienburg (1), Give (1), Agona (1), Cerro (1), Cubana (1), Muenster (1), Norwich (1), Anatum var. 15+ 34+ (1), Muenster var. 15+ 34+ (1), Schwarzengrund (1), Anatum var. 15+ (1), Senftenberg (1), Orion var. 15+ (1), Lille (1), and Braenderup (1). *E. coli* serogroups (no. of strains) used were O26 (30), O45 (4), O103 (41), O111 (39), O121 (17), O145 (10), O104 (16), O157 (4), O6 (3), O8 (19), O15 (2), O22 (1), O25 (1), O38 (3), O39 (3), O49 (1), O55 (1), O74 (3), O78 (2), O84 (3), O88 (3), O91 (2), O96 (3), O109 (3), O113 (3), O116 (3), O117 (3), O118 (2), O127 (1), O130 (4), O136 (3), O141 (3), O142 (3), O146 (1), O150 (1), O153 (2), O159 (1), O163 (3), O171 (3), and O172 (2). Other bacterial species tested included *Listeria monocytogenes* (1), *Streptococcus pyogenes* (1), *Enterobacter aerogenes* (2), *Serratia marcescens* (3), *Morganella morganii* (1), *Enterococcus*

faecium (1*), E. faecalis* (2), *E. casseliflavus* (1), *Klebsiella pneumoniae* (2), and *Proteus mirabilis* (2), *Proteus vulgaris* (2), *Actinobacillus pleuropneumoniae* (2), *Bordetella bronchiseptica* (2), *Mannheimia haemolytica* (2), *Pasteurella multocida* (2), *Pseudomonas aeruginosa* (2), *Pseudomonas stutzeri* (2), and *Histophilus somni* (1). Each strain was streaked onto BAP and incubated overnight at 37 °C. Three to five colonies of each culture were suspended in 1 mL nuclease free water, boiled for 10 min and centrifuged for 5 min at 9300 g. Supernatant was used as template for qPCR specificity testing.

3.5. Lymph nodes sample collection and preparation

A total of 647 subiliac lymph nodes were collected from cattle procured from Texas, Oklahoma, Kansas, and South Dakota (Cernicchiaro et al., 2016) and transported in cold storage to the Kansas State University Pre-Harvest Food Safety Laboratory for processing within 24 h. Each lymph node was manually trimmed to remove fat and fascia tissues. Trimmed lymph nodes were surface sterilized by a 5 s submersion in boiling water, placed in a sterile bag, then manually pulverized with a rubber mallet. Eighty milliliters of TSB were added to each bag and sterilized lymph nodes were then homogenized for 30 s in a Stomacher 80 Biomaster (Thomas Scientific, Swedesboro, NJ).

3.6. Pre-enrichment sample preparation for real-time PCR and culture isolation

One milliliter of the homogenate was removed for DNA extraction and qPCR amplification. One hundred microliters of the homogenate were also streaked onto Hektoen Enteric (HE, Becton, Dickinson Co., Sparks, MD) agar plates and cultured at 37 °C for 18–20 h. Single

colonies from the HE agar plates were sub-cultured onto BAPs and tested by *Salmonella*-specific agglutination assay.

3.7. Enrichment sample preparation for real-time PCR and culture

Salmonella enrichment procedure has been described (Brichta-Harhay et al., 2012; Cernicchiaro et al., 2016). Briefly, the remaining portion of the homogenate was incubated at 25 °C for 2 h then at 42 °C for 12 h. One milliliter of enriched homogenate was then subjected to immunomagnetic separation using 20 μL anti-*Salmonella* beads. One hundred microliters of PBS were added to the final immunomagnetic separation step. The bead suspension was then transferred into 3 mL RV broth and incubated at 42 °C for 18–20 h. One hundred microliters of enriched homogenate were streaked onto HE agar plates and incubated at 37 °C for 24 h. Six dark-colored colonies with morphology consistent with *Salmonella* were re-streaked onto BAPs and incubated at 37 °C for 18–20 h. The resulting cultures were subjected to an agglutination test with pooled *Salmonella* polyvalent O-antigen antiserum (Becton Dickinson, Franklin Lakes, NJ) for *Salmonella* confirmation. DNA was also extracted from enriched samples by the boiling method described above and subjected to qPCR.

3.8. Statistical analysis

The overall agreement between qPCR and culture methods was assessed by the Cohen's Kappa statistic and 95% confidence interval using the Kappa calculator http://vassarstats.net/kappa.html). Kappa statistic values were interpreted based on the scale proposed by Landis and Koch (Landis and Koch, 1977).

4. Results

4.1. Assay sensitivity with pure culture

Standard curves with 10-fold dilutions of pure cultures of three *Salmonella* serotypes were used to determine the analytical sensitivity of the assay. Colony-forming counts of the culture were 1.8×108, 6.4×107, and 4.3×107 CFU/mL, and detection limits of the multiplex real-time PCR were 1.8×104, 6.4×103, and 4.3×103 CFU/mL for *S.* Typhimurium, *S.* Anatum and *S.* Montevideo serotypes, respectively. For all strains tested, the average endpoint threshold cycle (Ct) ranged from 35.51 to 37.96 and 35.04 to 38.26 for *inv*A and *pag*C targets, respectively. Correlation coefficients were all >0.99, and PCR amplification efficiencies were between 93% and 110%. Figure 3.1 shows the standard curve generated for *S.* Typhimurium. Similar curves were also generated for the other two *Salmonella* serotypes (data not shown).

4.2. Standard curve and limit of detection with culture-spiked lymph node samples

Very similar detection limits were observed among the three *Salmonella* serotypes for both pre- and post-enriched lymph node spike in samples. For pre-enriched samples, detection limits for the two replications of lymph node preparations for S. Typhimurium, S. Anatum and S. Montevideo were 5.5×104 and 7.4×103 CFU/mL, 2.1×104 and 6×104 CFU/mL, and 3.6×104 and 4.7×104 CFU/mL, respectively. For all strains tested, the average endpoint threshold cycle (Ct) ranged from 35.10 to 37.32 and 34.85 to 37.84 for *inv*A and *pag*C targets, respectively. Correlation coefficients were all >0.99, and PCR amplification efficiencies ranged from 100% to 104%. Following enrichment, detection limits for the two replications were 5.5 and 0.74 CFU/mL, 0.21 and 0.6 CFU/mL, and 0.36 and 0.47 CFU/mL, for *S.* Typhimurium, *S.* Anatum,

and S. Montevideo, respectively (Table 3.2). Bacterial concentrations became more similar for different dilutions following the enrichment steps, and qPCR data for post-enrichment samples are end-point Cts, thus standard curves were not performed on these samples.

4.3. Sensitivity of spiked lymph nodes by culture method

Without the enrichment step, detection limits by HE plating were between 2.1×102 and 4.7×103 CFU/mL; average detection limits for the two replications were 6.5×102, 4.1×102 and 2.5×103 CFU/mL, for *S.* Typhimurium, *S.* Anatum, and *S.* Montevideo serotypes, respectively. The detection sensitivity was significantly increased by culture enrichment. The average detection limits for culture-based methods were the same as the values observed for qPCR after enrichment: 3.1 CFU/mL, 0.41 CFU/mL, and 0.42 CFU/mL, for *S.* Typhimurium, *S.* Anatum, and *S.* Montevideo serotypes, respectively (Table 3.2).

4.4. Assay specificity analysis

Our culture collection of 36 *Salmonella* strains, represent 33 serotypes, were all tested positive for *inv*A and *pag*C targets; the 10 feed *Salmonella* isolates and the 28 feed environmental isolates of unknown serotypes were also positive for both targets. All non-*Salmonella* strains, which included the 252 *E. coli* strains representing 40 different O-serogroups, and 31 other bacterial strains representing 18 different bacterial species, produced no signal for *inv*A and *pag*C targets.

4.5. Detection of *Salmonella* in lymph nodes of feedlot cattle by real-time PCR assay and culture method

Prior to culture enrichment, 19.8% (128/647) of lymph node tested positive for *Salmonella* by qPCR, and 7.7% (50/647) of lymph nodes were positive by culture method. Ninety-six percent (48/50) of culture positive samples were also positive by qPCR. A majority of the 128 qPCR positive samples were at concentrations between 104 (52/128; 40.6%) and 105 CFU/g (53/128; 41.4%). The remaining samples were at concentrations of 103 (6/128; 4.7%) and 106 (17/128; 13.3%) CFU/g. A large proportion of enriched cattle lymph nodes were positive for *Salmonella* by qPCR (614/647; 94.9%) and by culture method (522/647; 80.7%; Figure 3.2). Among the 522 culture positive samples, 519 (99.4%) were also positive by qPCR. The Cohen's Kappa statistics indicated almost perfect agreement between qPCR and culture methods for both pre-enriched (κ=0.98; 95% CI of 0.95–1.00) and enriched (κ=0.99; 95% CI of 0.97–1.00) sample detections. The Ct range after the enrichment was between 20.0 and 37.9 and peaks at Ct 26 (Figure 3.3A). The normal distribution of the Ct values prior to enrichment reflected the variable distribution of bacterial concentrations presented in the original lymph node samples (Figure 3.3B).

4.6. Variation between the *inv*A and *pag*C targeted

Based on standard curves, Ct 38 was the cutoff value for a sample to be considered positive (more in the Discussion section). Among preenriched samples, 20/128 were positive for *inv*A target only (*pag*C negative), and 13/128 positive for *pag*C only (*inv*A negative), all with Cts

of 35.4–37.9. Interestingly, all 33 single-target positive samples were strong positives for both targets following enrichment (data not shown). Ct values for all single target positive

samples were higher than 35, indicating that the variations between the two molecular targets occurred only when bacterial concentrations were low.

5. Discussion

The overall strain coverage of a molecular detection assay is largely dependent upon the conservation level of the molecular target selected (Bai et al., 2010; Shi et al., 2016). In general, the more sequences analyzed during the test design, the better the test coverage will be. However, scarcity of available sequences often limits the design of a detection assay. Advanced sequencing technology has generated a tremendous amount of sequence information in recent years. As a result, it may be necessary to re-design primer(s)/probe(s) of molecular detection assays developed prior to this period. Because collective mutation rate in two genes, in the given bacterial strain, is much lower than that on a single gene, the use of more than one conserved molecular targets might detect potential genetic variations better than by utilizing a single molecular target. Even though, a molecular assay needs to be monitored and reanalyzed periodically in order to maintain its diagnostic specificity, in particular, its diagnostic sensitivity that is best predicted by primer and probe coverages over available target sequences. This may be the most important strategy to maintain the effectiveness of a diagnostic assay against continued mutations in field populations of the pathogen.

Due to its conserved nature, the *inv*A gene has served as a common molecular target in many PCR-based *Salmonella* detection protocols (Barletta et al., 2013; Cunningham et al., 2010; Garrido et al., 2013; Li et al., 2014; Timmons et al., 2013; Zhang et al., 2013b). Based on our in silico analysis, the *pag*C gene is similarly conserved, however has been scarcely utilized in

Salmonella detection (Wang et al., 2018). In this study, we explored the advantages of targeting both the *inv*A and *pag*C genes for serotype non-specific detection of *Salmonella enterica*. During initial test development, partial *inv*A and *pag*C genes flanking the qPCR primers from 10 *Salmonella* strains were sequenced. Sequenced fragments matched with the GenBank *inv*A and *pag*C sequences.

All 33 single target positives identified in the pre-enriched samples had Ct values >35, indicating very low *Salmonella* concentrations in the samples. When bacterial concentration in a sample is high, template is readily available for primers and/or probe(s) to bind to, however, when fewer template copies are present, primer binding may become more randomized. Since the *inv*A and *pag*C genes are separated by more than one million base pairs on the *Salmonella* genome and because the genomes were fragmented into <50 kb segments during DNA extraction, it is unlikely that the two targets were located on the same DNA fragment. Therefore, only a single target may have been present in a given qPCR reaction when *Salmonella* DNA concentration in a sample was low. This is further supported by enriched sample data in which all 33 single-target positive samples were qPCR positive to both targets, and 32 of the 33 samples were also positive by the culture method.

Limit of detection (LOD) values are commonly determined by standard curves. In this study we have generated six standard curves (3 serotypes with 2 replications), each with three replications for both culture and culture-spiked lymph nodes. Therefore, two sets of 18 data points were generated for LOD determinations. The LOD Cts for *inv*A were 35.51–37.96 for culture, and 35.10–37.32 for spiked lymph nodes; and for *pag*C were 35.04–38.26 for culture and 34.85–37.84 for spiked lymph nodes. Although the mean LOD Cts were 36.15 and 36.21 for the two genes, we still used Ct 38 as cutoff LOD for both genes for two main reasons: 1) The

real Ct cutoff may occur in between two 10-fold dilutions, therefore the prepared dilutions may not reflect the true cutoff value. For example, a sample may generate a Ct of 35.7 for a dilution and an expected Ct of 39.0 for the next dilution (10-fold dilution=3.3 Ct difference). However, if no amplification is observed for the second dilution, 35.7 would be considered the cutoff even though the true cutoff Ct can be anywhere in between 35.7 and 39.0; and 2). We specifically checked the data for the 33 pre-enriched single target-positives. Thirty one of the 33 samples had Cts of 36.0–38.0 including six that were Ct 37.9 (with Ct cutoff of 38). Yet all 33 samples were tested strong positive by qPCR on both targets after the enrichment; and 32/33 were true positives defined by culture-positive status following the enrichment. Therefore, although the calculated Ct cutoff was 36.2, Ct 38 may reflect the real LOD Ct cutoff in identifying positive *Salmonella* samples.

Our data indicated higher *Salmonella* prevalence rates by both qPCR and culture methods compared to other studies (Arthur et al., 2008; Brichta-Harhay et al., 2012), however similarly high prevalence rates have also been reported (Cernicchiaro et al., 2016; Gragg et al., 2013b). Variable results were observed for *Salmonella* carriage by lymph node by different factors including geographical locations (Haneklaus et al., 2012; Webb et al., 2017), season (Arthur et al., 2008; Brown et al., 2015; Gragg et al., 2013a; Webb et al., 2017), and breed (Brown et al., 2015). More pre- and post-enriched samples were positive by qPCR than by culture method, indicating that the qPCR, as compared to the culture method, is a more sensitive one for the detection of *Salmonella* from cattle lymph nodes samples. This qPCR with increased detection sensitivity may be helpful in generating more accurate data for future studies on *Salmonella* prevalence in cattle lymph nodes.

Salmonella concentrations of pre-enriched positive samples were estimated based on the Ct values by qPCR. A majority of these positive samples (n=128) were at concentrations of 104 (52/128; 40.6%) and 105 CFU/g (53/128; 41.4%) (Figure 3.3B). Although the majority of enriched samples (445/647; 68.8%) had Cts between 24 and 29, 10.5% (68/647) of samples had Cts of 20–23, and 15.6% (101/647) of samples had Cts between 30 and 37 (Figure 3.3A).

For several of our *E. coli* detection assays (Bai et al., 2010, 2012; Noll et al., 2015; Shridhar et al., 2016), PCR-based methods were significantly more sensitive than culture methods. In this study, we observed similar sensitivities between qPCR and culture as based on Kappa analysis. Most *Salmonella* enrichment procedures, like the one used in this study, utilize two enrichment steps (instead of one) and some also include a *Salmonella*-specific immunomagnetic bead separation step. These additional steps greatly increased detection sensitivity and may have contributed to similar sensitivities observed between culture and qPCR methods. However, qPCR method was still more sensitive; furthermore, results from the qPCR procedure are generated at least 2 days sooner compared to the more time-consuming culture method of detection. Among the 128 pre-enrichment samples positive for *Salmonella* by qPCR, 128 and 127 of the samples were also positive by qPCR following the enrichment and by culture method, respectively, confirming that qPCR positive samples were true positives; some PCR products were also confirmed by sequencing. The inclusion of the 18S rRNA internal control further increased the accuracy of the assay by eliminating potential false-negative detections (Bai et al., 2018, submitted).

Several PCR-based methods have been developed for *Salmonella* detection. Our in silico analysis identified some nucleotide mismatches between the primer sequences published in the literature and *Salmonella* sequences available in the GenBank database at the time of our assay

design. Advancement of new technology like next generation sequencing has markedly increased availability of sequence information, including for *Salmonella*, and has made it necessary to periodically confirm that previously published primer(s)/probe(s) sequences are still providing adequate detection of target organisms in the population. In this study, 15.6% (20/128) and 10.2% (13/128) of pre-enriched samples were single-target positive for *inv*A and *pag*C, respectively. Other assays targeting *inv*A alone may not have detected the 10.2% of samples positive for *pag*C and negative for *inv*A, and single target positives by our assay should be considered true positives. Furthermore, the use of two targets for detection of an organism may safeguard against potential gene mutations, as mutations on both targets on the same strain is less frequent. Therefore, this assay may be more useful compared to previous assays for the detection of *Salmonella* enterica strains.

In conclusion, we have developed a triplex real-time qPCR assay for the detection and quantification of *Salmonella* strains in cattle lymph node samples. As *Salmonella*-containing lymph nodes become an increasing food-safety concern, this assay should prove to be a useful tool to detect and monitor prevalence of the organism in cattle lymph nodes, and potentially in other sample types.

Tables and figures

Table 5-1. Primer and probe information for the three genes in the triplex real-time PCR.

Salmonella gene	Name	Sequence	Amplicon size (bp)	Location on M555461.1 (*pag*C) or DQ644626.1 (*inv*A) or DQ222453.1 (18S)	Number/total sequences (percentage) that have perfect matches
*pag*C	S*pag*C-F2	5′-AAGTTGATGGTGCGAGGTTC-3′	90	1738–1757 nt	549/549 (100%)
	S*pag*C-R2	5′-CCACATCATCAGCCTGACAC-3′		1827–1808 nt	533/549 (97.1%)
	S*pag*C-Pr2	5′-MAX-TGGCGGTCCAATGTGGTTAT-BHQ1-3′		1767–1787 nt	543/549 (98.9%)
*inv*A	S*inv*A-F	5′-CGTGTTTCCGTGCGTAATA-3′	138	1631–1649 nt	613/615 (99.7%)
	S*inv*A-R	5′-GCCATTGGCGAATTTATG-3′		1768–1751 nt	614/615 (99.8%)
	S*inv*A-Pr	5′-FAM-ATTATGGAAGCGCTCGCATT-BHQ1–3′		1658–1677 nt	612/613 (99.8%)
18S rRNA	18S-F	5′-GGAGTATGGTTGCAAAGCTGA-3′	100	1168–1188 nt	89/89 (100%)
	18S-R	5′-GGTGAGGTTTCCCGTGTTG-3′		1267–1249 nt	88/89 (98.9%)
	18S-Pr	5′-Cy5-AAGGAATTGACGGAAGGGCA-BHQ2–3′		1195–1214 nt	89/89 (100%)

F: Forward primer; R: Reverse primer; Pr: Probe; FAM: Fluorescein, or Fluorescein amidate;

MAX: Rhodamine (VIC equivalent); Cy5: Cyanine 5; BHQ: Black-Hole Quencher

Table 5-2. Maximun detection limits for *Salmonella* pure culture and culture-spiked cattle lymph nodes using real-time PCR and culture method of detection.

	Real-time PCR						Culture					
	Spiked lymph nodes (CFU/ml)			Spiked lymph nodes (CFU/ml)			HE plate (CFU/ml)			HE plate (CFU/ml)		
	Pre-enrichment			Post-enrichment			Pre-enrichment			Post-enrichment		
	Replicates			Replicates			Replicates			Replicates		
	1	2	Mean	1	2	Mean	1	2	Mean	1	2	Mean
S. Typhimurium	5.5×10^{4}	7.4×10^{3}	3.1×10^{4}	5.5	0.74	3.1	5.5×10^{2}	7.4×10^{2}	6.5×10^{2}	5.5	0.74	3.1
S. Anatum	2.1×10^{4}	6×10^{4}	4.1×10^{4}	0.21	0.6	0.41	2.1×10^{2}	6.0×10^{2}	4.1×10^{2}	0.21	0.6	0.41
S. Montevideo	3.6×10^{4}	4.7×10^{4}	4.2×10^{4}	0.36	0.47	0.42	3.6×10^{2}	4.7×10^{3}	2.5×10^{3}	0.36	0.47	0.42
Average			3.77×10^{4}			1.32			1.19×10^{3}			1.32

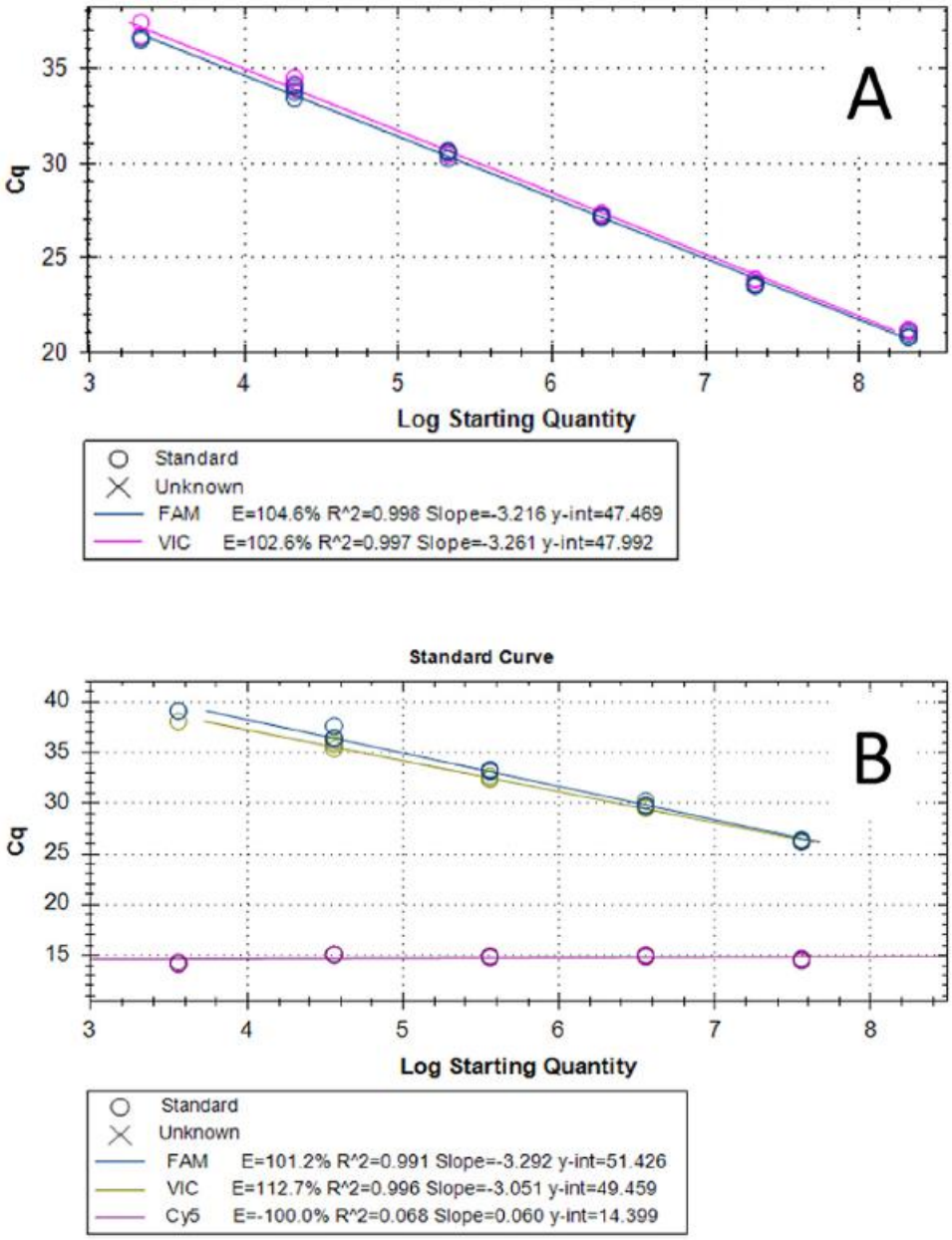

Figure 5-11. Standard curves. Panel A: standard curve detection of *inv*A (FAM channel; blue) and *pag*C (VIC/MAX channel; pink) genes of serially diluted *S.* Typhimurium culture. Panel B: Standard curve detection of *inv*A (FAM, blue) and *pag*C (VIC/MAX, yellow) with the inclusion of 18S rRNA gene (Cy5, purple) as internal control using a lymph node spiked with 10-fold dilutions of a *S.* Typhimurium culture. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

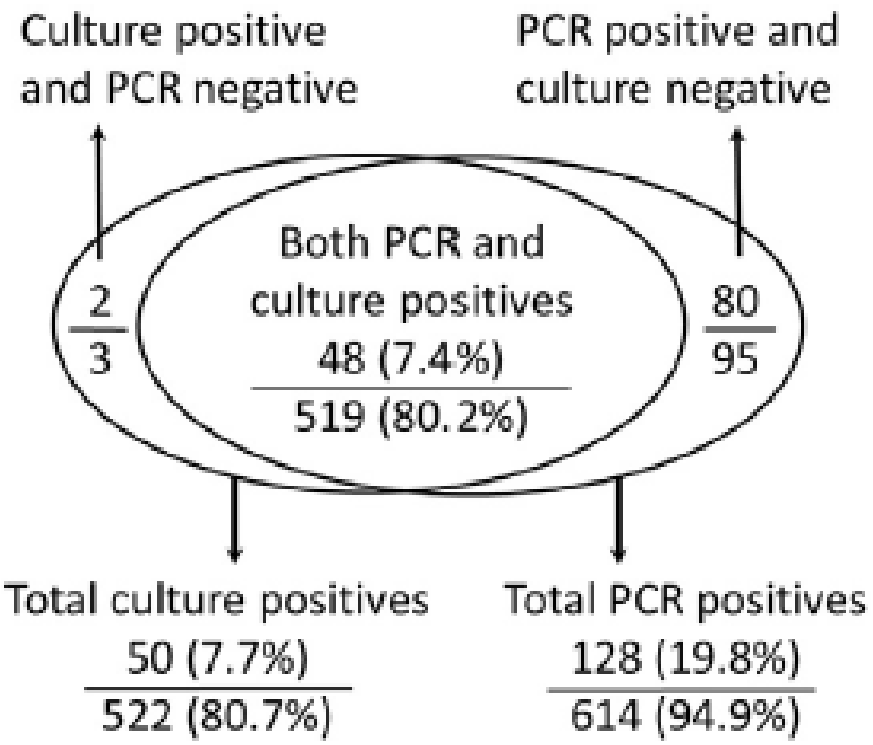

Figure 5-12. *Salmonella* positive rates from pre- and post-enrichment cattle lymph node samples (n=647) tested by the multiplex real-time qPCR and the traditional culture method. Number (%) above the line: pre-enrichment data; Number (%) below the line: post-enrichment data.

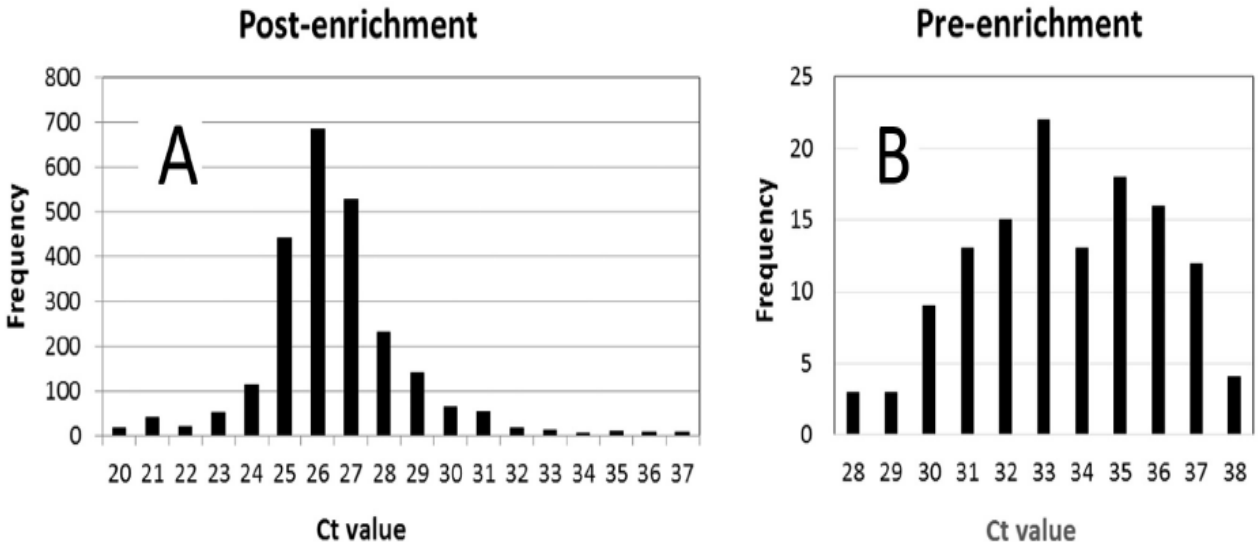

Figure 5-13. Real-time qPCR Ct distribution and frequency of cattle lymph nodes samples positive for *inv*A and *pag*C genes. Panel A: 614 positive samples (of 647 total) after enrichment; Panel B: 128 positive samples before enrichment.

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Appendix C - SAS codes for statistical analysis

Fall samples with culture positive (C+) outcome

```
PROC IMPORT OUT=feedmill DATAFILE="C:\Users\Administrator\Desktop\dataset.xlsx"
        DBMS=xlsx REPLACE;
    SHEET="Fall";
            GETNAMES=YES
```

Univariable models

```
proc glimmix data=feedmill method=laplace;
class state mill_name mill_location mill_type month sample_site sample culture pcr_inva
sample_id;
model culture(desc)=sample_site/dist=binary link=logit;
random int/subject=mill_name;
random int/subject=mill_type;
random int/subject=month;
nloptions tech=nrridg;
lsmeans sample_site/ilink cl pdiff=all adj=tukey;
run;


proc glimmix data=feedmill method=laplace;
class state mill_name mill_location mill_type month sample_site sample culture pcr_inva
sample_id;
model culture(desc)=mill_type/dist=binary link=logit;
random int/subject=sample_site;
random int/subject=state;
random int/subject=mill_name;
random int/subject=month;
nloptions tech=nrridg;
lsmeans mill_type/ilink cl pdiff=all adj=tukey;
run;

proc glimmix data=feedmill method=laplace;
class state mill_name mill_location mill_type month sample_site sample culture pcr_inva
sample_id;
model culture(desc)=mill_name/dist=binary link=logit;
random int/subject=sample_site;
nloptions tech=nrridg;
```

```
lsmeans mill_name/ilink cl pdiff=all adj=tukey;
run;


proc glimmix data=feedmill method=laplace;
class state mill_name mill_location mill_type month sample_site sample culture pcr_inva
sample_id;
model culture(desc)=state/dist=binary link=logit;
random int/subject=sample_site;
random int/subject=mill_name;
nloptions tech=nrridg;
lsmeans state/ilink cl pdiff=all adj=tukey;
run;
```

Feed mill samples with PCR with molecular positive (PCR+) as outcome

```
PROC IMPORT OUT=feedmill DATAFILE= " C:\Users\Administrator\Desktop\dataset.xlsx"
        DBMS=xlsx REPLACE;
    SHEET="no triplicates";
    GETNAMES=YES;
RUN;
```

Univariable models

```
proc glimmix data=feedmill method=laplace;
class mill_name mill_type month visit sample_site sample culture pcr_inva sample_id
salmonella;
model salmonella(desc)=sample_site/dist=binary link=logit;
random int/subject=mill_name;
random int/subject=visit;
nloptions tech=nrridg;
lsmeans sample_site/ilink cl pdiff=all adj=tukey;
run;

proc glimmix data=feedmill method=laplace;
class mill_name mill_type month visit sample_site sample culture pcr_inva sample_id
salmonella;
model salmonella(desc)=visit/dist=binary link=logit;
random int/subject=sample_site;
random int/subject=mill_name;
nloptions tech=nrridg;
```

```
lsmeans visit/ilink cl pdiff=all adj=tukey;
run;

proc glimmix data=feedmill method=laplace;
class state mill_name mill_location mill_type month visit sample_site sample culture pcr_inva
sample_id salmonella;
model salmonella(desc)=mill_type/dist=binary link=logit;
random int/subject=sample_site;
random int/subject=state;
random int/subject=mill_name;
random int/subject=visit;
nloptions tech=nrridg;
lsmeans mill_type/ilink cl pdiff=all adj=tukey;
run;

proc glimmix data=feedmill method=laplace;
class state mill_name mill_location mill_type month visit sample_site sample culture pcr_inva
sample_id salmonella;
model salmonella(desc)=mill_name/dist=binary link=logit;
random int/subject=sample_site;
random int/subject=visit;
nloptions tech=nrridg;
lsmeans mill_name/ilink cl pdiff=all adj=tukey;
run;
```

Multivariable model

```
proc glimmix data=feedmill method=laplace;
class state mill_name mill_location mill_type month visit sample_site sample culture pcr_inva
sample_id salmonella;
model salmonella(desc)=mill_name visit sample_site/dist=binary link=logit s cl;
nloptions tech = nrridg;
lsmeans mill_name visit /ilink cl pdiff=all adj = tukey;
run;
```

Multivariable interactions

```
proc glimmix data=feedmill method=laplace;
class state mill_name mill_location mill_type visit sample_site;
model salmonella(desc)=mill_name|visit /dist=binary link=logit s cl;
random int/subject=sample_site;
nloptions tech = nrridg;
```

```
lsmeans mill_name visit mill_name*visit /ilink cl pdiff=all adj = tukey;
run;

proc glimmix data=feedmill method=laplace;
class state mill_name month mill_location mill_type visit sample_site;
model salmonella(desc)=sample_site|visit /dist=binary link=logit s cl;
nloptions tech = nrridg;
lsmeans sample_site visit sample_site*visit /ilink cl pdiff=all adj = tukey;
run;

proc glimmix data=feedmill method=laplace;
class state mill_name month mill_location mill_type visit sample_site;
model salmonella(desc)=sample_site|mill_name /dist=binary link=logit s cl;
random int/subject=visit;
nloptions tech = nrridg;
lsmeans sample_site mill_name sample_site*mill_name /ilink cl pdiff=all adj = tukey;
run;
```

Appendix D - Map of the states where feed mills were selected in this study.

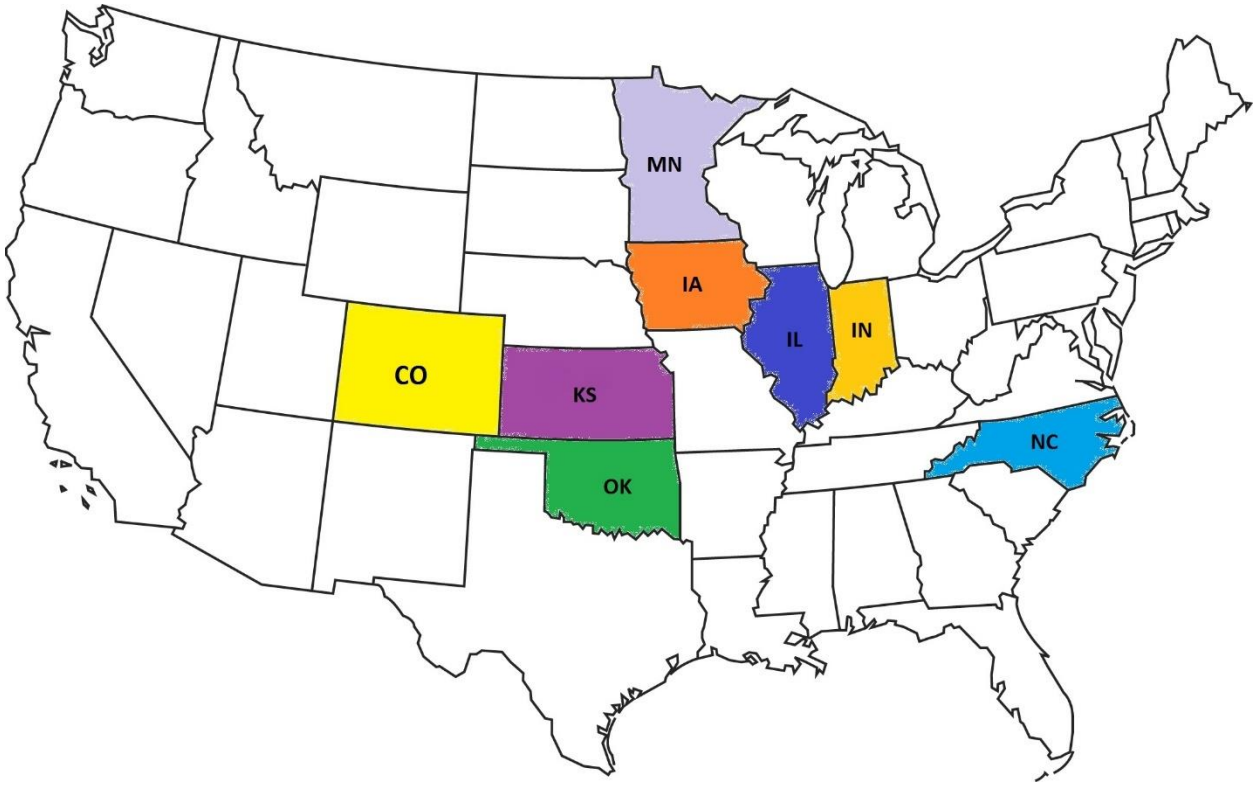

Figure C-1. Map of the United States with states where feed mills were located highlighted

Appendix E - Bacterial diversity of environmental feed mill and swine feed samples other than *Salmonella* spp.

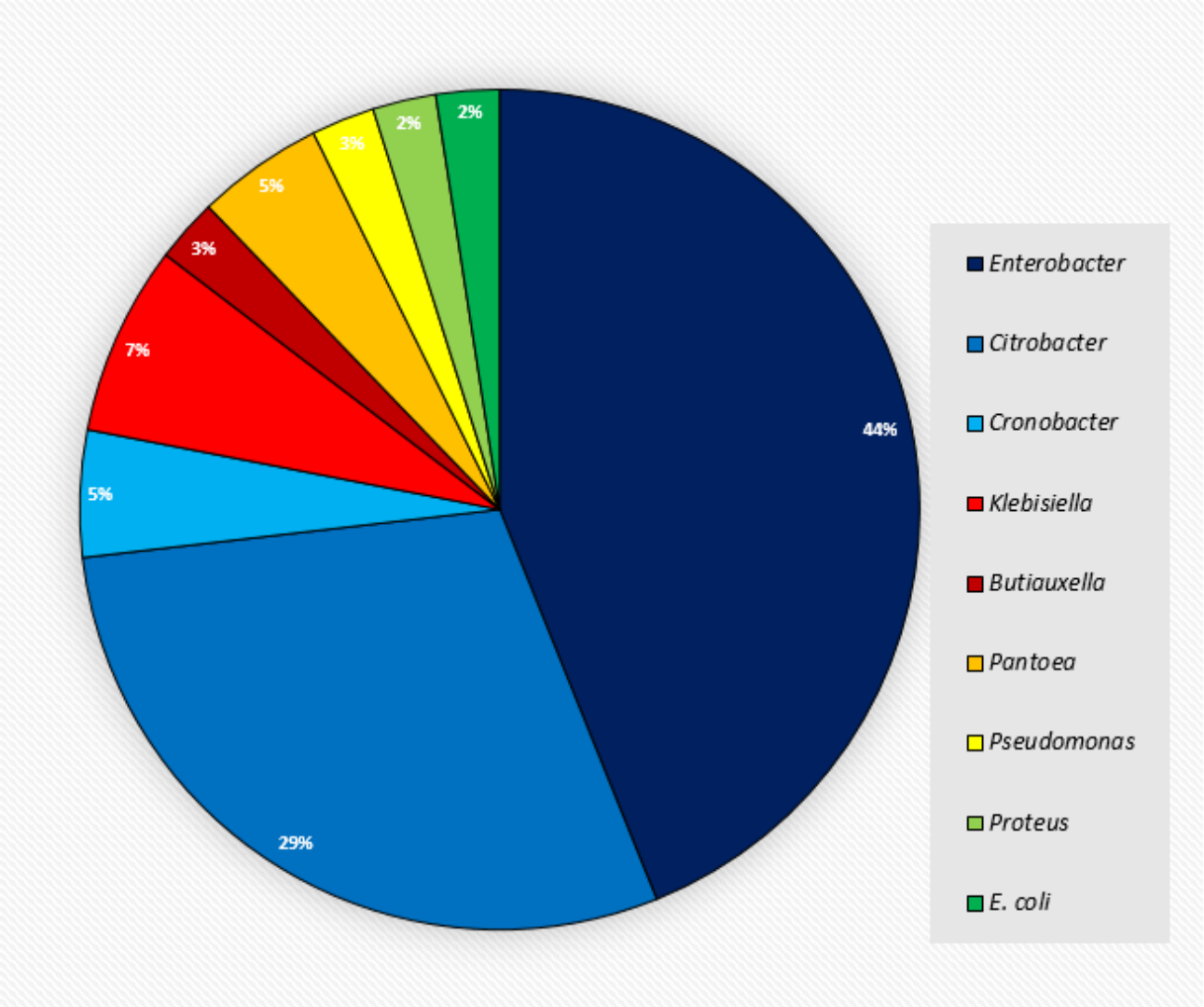

Figure D-1 Bacterial genus of samples culture positive but PCR negative for *Salmonella* spp.

Appendix F - Poster presented at scientific conference

Investigation of the Presence of *Salmonella spp.* in United States Feed Mills

G. Magossi[1], N. Cernicchiaro[2], S. Dritz[3], T. Houser[4], J. Woodworth[3], C. Jones[4], and V. Trinetta[1]

[1]Food Science Institute, [4]Department of Animal Sciences and Industry, [2]Diagnostic Medicine and Pathobiology, [3]College of Veterinary Medicine, Kansas State University

Introduction

- According to the CDC, each year *Salmonella* causes one million foodborne illnesses in the United States, with 19,000 hospitalizations and 380 deaths (CDC, 2016).
- *Salmonella* is a resilient microorganism that can survive for long periods of time in low water activity (Podolak et al., 2011).
- *Salmonella* has been linked to numerous outbreaks associated with pork products and it has been previously isolated from swine feed and feed production facilities (Abrahantes et al., 2009).
- Bacteria present in animal feed can transfer to animals and find their way into the human food supply chain (Crump et al., 2002).
- Understanding pathogen presence in feed mill environments can be key to implement control programs at pre-harvest level (Rostagno and Callaway, 2012).

Objectives

1) Determine the presence and distribution of *Salmonella spp.* in commercial mills manufacturing animal feed.
2) Characterize the distribution of *Salmonella* positive isolates in relation to sampling locations and establishment- production associated risk factors.

Materials and Methods

Swabbing

Eleven feed mill locations in eight states were selected for this study (Figure 1). Environmental samples were collected with a sterile sponge-stick pre-wet with 10ml of buffered peptone water. Floor surfaces were swabbed using a 10cmX10cm template in triplicates. Worker shoes samples were collected from both left and right shoes. Finished feed samples were collected from fresh feed manufactured the same day of the visit. Swabbing sites are listed in table 1.

Phenotypical analysis

Phenotypical analysis was conducted following the USDA-FSIS laboratory guidebook for the isolation and identification of *Salmonella* from meat, poultry, pasteurized egg catfish products carcass and environmental sponges (FSIS, 2011).

Agglutination

Presumptive positive isolates from the phenotypical analysis were further analyzed with serological typing methods. A *Salmonella* O antiserum polyvalent for groups A through G + iv (BD Difco *Salmonella* O Antisera, Sparks, MD) in slide agglutination tests was used for the identification of *Salmonella* somatic (O) antigens.

PCR confirmation

Samples that were positive for phenotypical analysis and agglutination tests were further confirmed with real-time PCR, with an internal protocol, targeting the invasion gene *invA* present in *Salmonella* species.

Acknowledgement

The authors wish to thanks the National Pork Board for the funding support.

Results

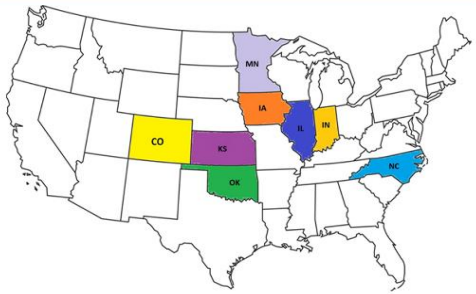

Figure 1. Feed mill locations selected for the study

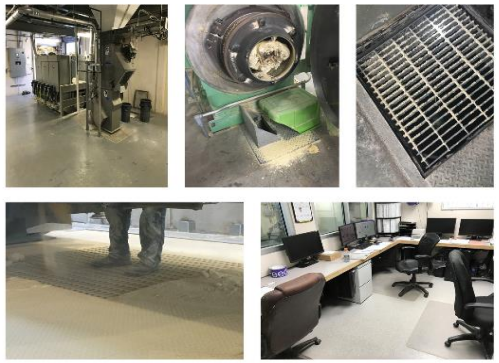

Figure 2. Selected swabbing sites within feed mill

Table 1. Prevalence of *Salmonella* spp. in environmental samples from 11 different feed mill locations – Fall 2016

	Feed mill										
	Pellet						Mash				
	KS-B	CO	OK	NC-A	IA-B	NC-B	KS-A	MN	IA-A	IN	IL
Ingredient pit grating[1]	+++	-+	++[5]	+++	+++	+++	+	+++	-+-	+++	+-+
Fat intake inlet	-	-	-	*	-	+	-	-	-	+	-
Exterior of pellet mill	-	-	-	+	-	+	n/a	n/a	n/a	n/a	n/a
Finished product bin boot	-	+	+	+	+	+	+	+	-	+	+
Load-out auger	-	-	-	-	+	+	+	+	-	+	-
Finished feed	-	-	-	+	-	+	+	+	-	+	--[3]
Floor dust in break or control room[1]	-+-	-++	+--	+++	-++	+--	+	+++	---	+++	+++
Floor dust in receiving[1]	+--	---	+++	+++	+++	+++	+	+++	+-+	+++	+++
Floor dust in manufacturing area[1]	+--	---	-+-	+++	---	+++	+	+++	++-	+++	-++
Floor dust in warehouse[1]	---	+-+	---	+--	-++	++-	-	-+-	-++	+++	+++
Worker shoes[2]	++	-+	++	++	++	++	+	++	++	++	++
Broom	-	+	-	+	-	+	+	+	-	+	+

[1] Site sampled in triplicate
[2]Two different finished feed analyzed
[3] Site not present
[4]Left and right shoes
[5]Site could not be sampled
[6]Bold: PCR positive samples

Results

- Of the total 237 samples collected in fall 2016, 65.4% (155/237) tested positive for *Salmonella* spp. on selective media cultures (Table 1).
- Approximately 77% (34/44) of sites tested positive for *Salmonella* spp. in mash mills, whereas 66.3% (55/82) were positive in pellet mills.
- Across states *Salmonella* spp. presumptive positive samples ranged from 39.1 to 100%
- Among the presumptive positive samples, 11.8% (28/137) were confirmed *Salmonella* spp. by PCR (Table 1).
- Sites corresponding to floor dust in the manufacturing area, exterior of pellet mill, ingredient pit grating, floor dust in receiving area and floor dust in brake/control room showed the greatest percentage of confirmed positive samples (Figure 2) .

Conclusion and future research

The results of this study demonstrate the presence of *Salmonella* in feed mills environments across the United States and indicate their potential role of feed as a vehicle for pathogen transmission and spread into the food production chain. Further epidemiological studies will be necessary to asses persistency and seasonality of this pathogen.

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Figure E-1. Poster presented at the International Association for Food Protection annual meeting in Tempa-FL on July, 2017.